VOL. 38 OCTOBER, 1951 No. 4

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The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

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Vol. 38, No. 4, October, 1951. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription Price: United States, its possessions, Pan-American Countries, $10.00 per year; Canada, $11.00; Foreign, $11.00. Subscriptions to medical students, interns and residents, half price. Entered as Second-Class Matter, October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

IN VIVO OBSERVATIONS OF THE EFFECTS OF CORTISONE ON THE VASCULAR REACTION TO LARGE DOSES OF HORSE SERUM USING THE RABBIT EAR CHAMBER TECHNIQUE

ROBERT H. EBERT, M.D., AND ROBERT W. WISSLER, M.D.
CHICAGO, ILL.

SEVERAL investigators have reported that cortisone and ACTH modify the vascular lesions which develop in rabbits following large intravenous injections of foreign protein. Rich and co-workers[1, 2, 3] reported a reduction in the incidence of vascular and cardiac lesions in rabbits treated with ACTH or cortisone. Seifter and associates[4] reported similar results with cortisone, although they found that kidney lesions were not prevented. Rich and associates[2] felt that the kidney lesions usually associated with serum disease were inhibited, but described a different type of severe glomerular damage following treatment with cortisone but not with ACTH.

A previous communication from this laboratory[5] described certain in vivo changes which occurred consistently following large intravenous injections of normal horse serum in the rabbit. These changes were evaluated by repeated observations following serum injections using the rabbit ear chamber technique. It was felt that the vascular changes observed in vivo were a part of the fundamental vascular response leading to the arteritis which develops in serum sickness. It was suggested that the method might be useful in determining the mode of action of certain drugs.

The purpose of the present study was to determine the effect of cortisone on the vascular and intravascular changes which can be observed in vivo following large intravenous injections of normal horse serum, using the rabbit ear chamber.

METHODS AND MATERIALS

In general the methods were the same as described in the earlier paper.[5] Seven cortisone-treated animals were studied. Four of these animals had chambers in both ears so that the reaction was observed in eleven chambers. Body weight varied between 3,500 to 5,150 Gm., with an average of 4,500 Gm. Two intravenous injections of normal horse serum were given to all animals eighteen days apart (10 ml. per kilogram of body weight).

In the earlier report[5] it was noted that the most intense and consistent changes were seen during the first four days following the second injection of horse serum. Therefore, five animals were given 25 mg. of cortisone* daily intramuscularly in two divided doses for five days prior to the second injection of horse serum and for one to

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This work was sponsored by a grant from the United States Public Health Service.
Received for publication, June 27, 1951.
*Merck—Cortone Acetate; saline suspension.

three days after it. Two animals received 5 mg. of cortisone daily in one injection from the time of the first injection of horse serum until four days after the second.

Four of the seven animals were given a third injection of normal horse serum after cortisone therapy had been stopped so that a comparison could be made of the reaction in the same animal with and without treatment.

The various schedules of injections and treatment are diagramed in Fig. 1.

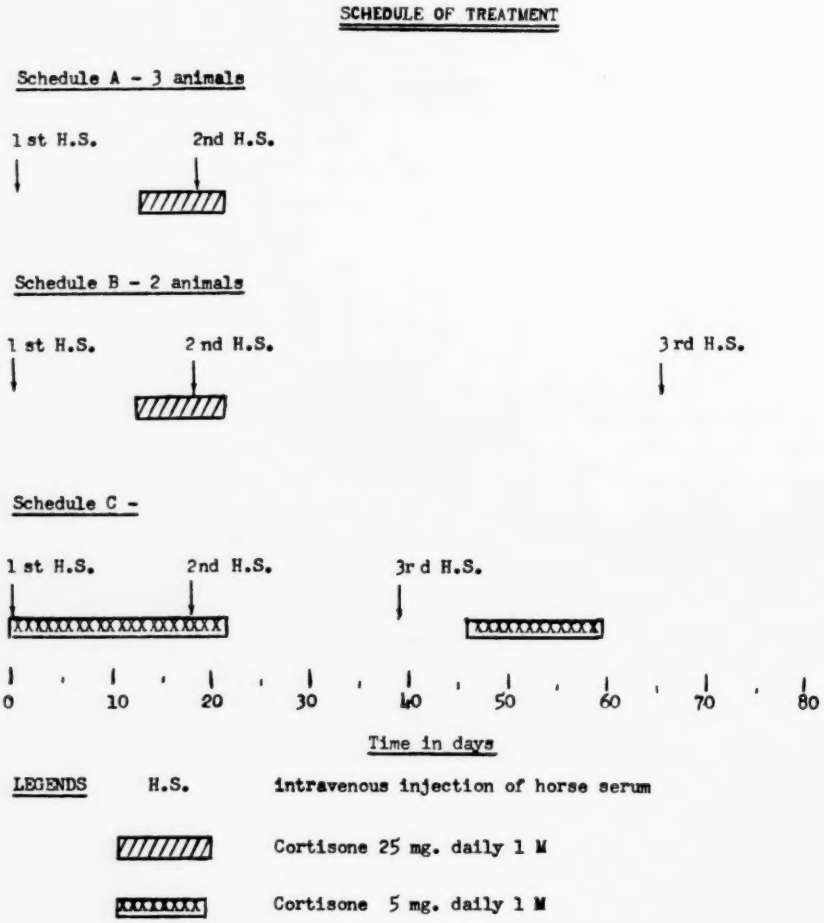

Fig. 1.

An attempt has been made to quantitate changes in arteriolar tone, sticking of leucocytes to arteriolar endothelium, and sludging with and without cortisone treatment (Figs. 3 and 4), as well as numbers of platelet and white blood cell thrombi and emboli (Fig. 7), and the same criteria have been used as in the previous paper.[5] The extent of these reactions was not only observed and recorded at the time they were taking place, but serial Kodachrome motion pictures were taken and carefully studied in an effort to evaluate the changes which occurred.

In addition a comparison was made between the systemic reactions in the six untreated animals previously described[5] and seven treated animals following the second injection of horse serum (Fig. 8). The degree of reaction was measured on an arbitrary

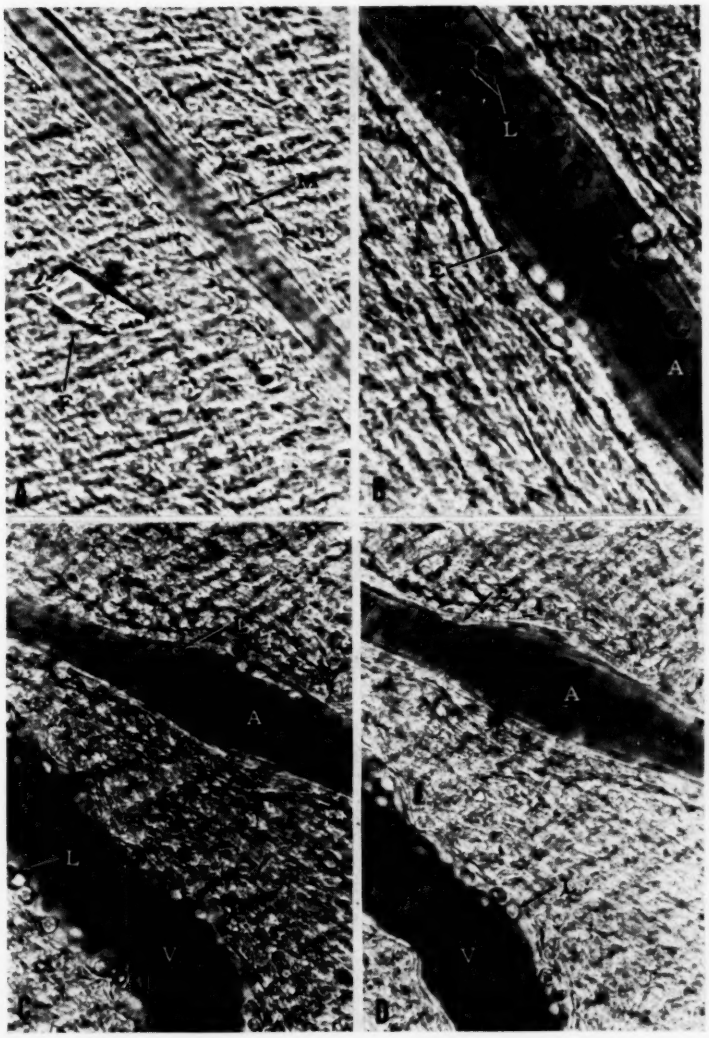

Fig. 2.—*A* = arteriole; *F* = foreign body; *L* = leucocyte; *E* = endothelium; *V* = venule; *M* = muscle.

A, Tone of arteriole (*A*) well maintained two hours after the second injection of horse serum in animal treated with cor isone rom the time of the first injection of horse serum. This is the same segment of arteriole shown in Fig. 5—adjacent to foreign body (*F*). Note absence of leucocyte sticking. (×400.) *B*, Another segment of the same arteriole (*A*) shown in Fig. 2*A* six hours after the third injection of horse serum. No cortisone for seventeen days. The arteriole (*A*) is dilated and there is sticking of leucocytes (*L*) to endothelium (*E*) (×400.) *C*, An arteriole (*A*) and venule (*V*) six days after the third injection of horse serum just before starting cortisone the second time (see schedule C, Fig. 1). There is sticking of leucocyte (*L*) in both the arteriole (*A*) and venule (*V*) (×400.) *D*, The same area as shown in Fig. 2*C* after two days of cortisone trea ment. Sticking of leucocytes (*L*) has disappeared from the arteriole (*A*) but persist in the venule (*V*), a localized dilatation of the arteriole persists but disappeared two days later. (×400.)

scale of 1+ to 4+, depending upon the amount of dyspnea, defecation, and urination which occurred during the second injection. Fig. 8 is an average of the reactions which occurred in treated and untreated animals.

RESULTS

I. *Vascular Damage.*—

A. *Changes in arteriolar tone:* The most striking effect of cortisone was upon arteriolar tone (compare Figs. 2*A* and 2*B*). Even in the apparently normal animal there was a gradual increase of tone during cortisone therapy, and changes in tone usually associated with serum sickness[5] were suppressed, if not completely abolished. Localized constrictions and dilatations present following the first injection of horse serum disappeared three to four days after starting cortisone treatment, and in the two animals treated from the time of the first injection (Fig. 3) no abnormalities occurred. In one of these

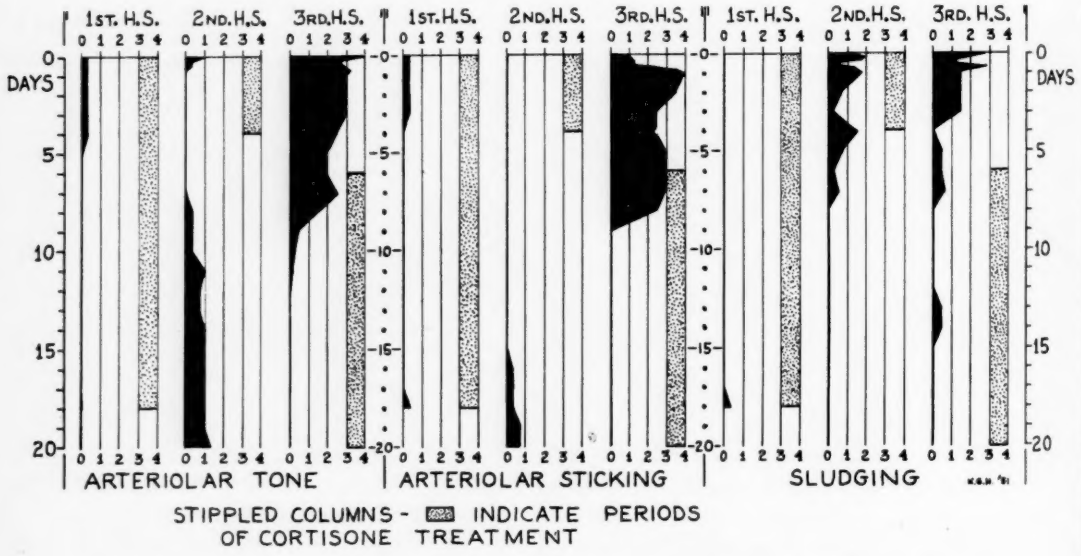

Fig. 3.—Serum sickness. Average of the reactions in two animals (three chambers) with and without cortisone treatment after first, second, and third injections of horse serum.

two animals (Fig. 3) treated from the first day of the experiment, the main arteriole supplying the observation table was abnormal prior to the first injection. Several localized dilatations were present in one area and two to three leucocytes were adherent to endothelium in the same region. It returned to normal on the fifth day of the experiment at a time when changes due to serum sickness might be expected to occur and remained normal until after the second injection of horse serum.

In only one of the seven treated animals there was cessation of blood flow on the observation table during the second injection of horse serum. In three of these animals given a third injection of horse serum after cortisone was stopped there was cessation of flow during the third injection. A fourth

animal given a third injection was not observed during the injection. It would appear that cortisone at least partially inhibited the spasm of larger arteries supplying the observation table which always occurred in untreated animals.[5]

The striking dilatation of arterioles following the second injection of horse serum and the subsequent development of localized constrictions and dilatations were quantitatively reduced by cortisone therapy (Figs. 3 and 4). In all cortisone-treated animals arterioles tended to maintain a more normal tone after the second injection. Although slight localized constrictions and dilatations sometimes developed, they were never as prominent as in untreated animals. After cortisone therapy was stopped tone was gradually lost and significant abnormalities developed. These changes are well illustrated in pho-

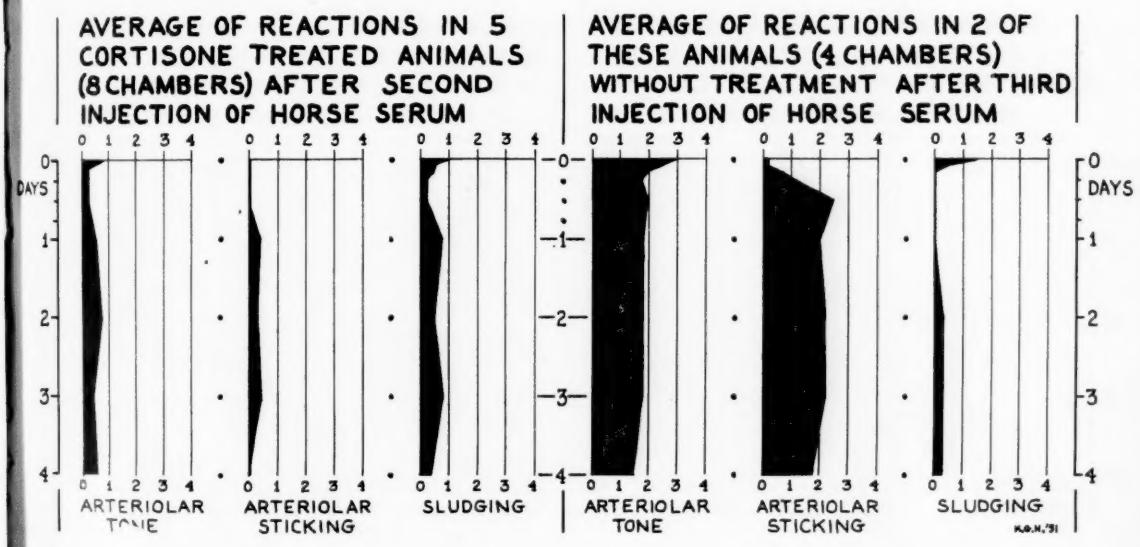
AVERAGE OF REACTIONS IN 5 CORTISONE TREATED ANIMALS (8 CHAMBERS) AFTER SECOND INJECTION OF HORSE SERUM
AVERAGE OF REACTIONS IN 2 OF THESE ANIMALS (4 CHAMBERS) WITHOUT TREATMENT AFTER THIRD INJECTION OF HORSE SERUM
DAYS
0 1 2 3 4
ARTERIOLAR TONE
ARTERIOLAR STICKING
SLUDGING
ARTERIOLAR TONE
ARTERIOLAR STICKING
SLUDGING

Fig. 4.—Comparison of serum sickness in the same animals with and without cortisone treatment.

tographs of the same segment of arteriole at comparable times after the second and third injections of horse serum (Fig. 5). This animal received cortisone from the first day of the experiment (Fig. 1), and it is apparent that following the second injection of horse serum arteriolar tone was well maintained (Figs. 5*A* and 5*C*) until cortisone treatment was stopped. Then there was gradual dilatation (Figs. 5*E* and 5*G*) which was accentuated after the third injection of horse serum (Figs. 5*B* and 5*D*). Tone was once again regained, however, upon resuming cortisone therapy (Figs. 5*F* and 5*H*). It is evident from these illustrations that not only is cortisone capable of suppressing this particular manifestation of serum sickness but can reverse the process once the reaction has occurred.

B. *Damage to arteriolar endothelium:* Sticking of leucocytes to arteriolar endothelium did not develop following the first injection of horse serum in

the two animals treated with cortisone from the first day of the experiment (Fig. 3). In one animal minimal arteriolar sticking was present prior to the first injection, and this disappeared after four days of treatment (Fig. 3). Sticking of leucocytes present in two of the five animals receiving cortisone for five days prior to the second injection disappeared after three days of therapy.

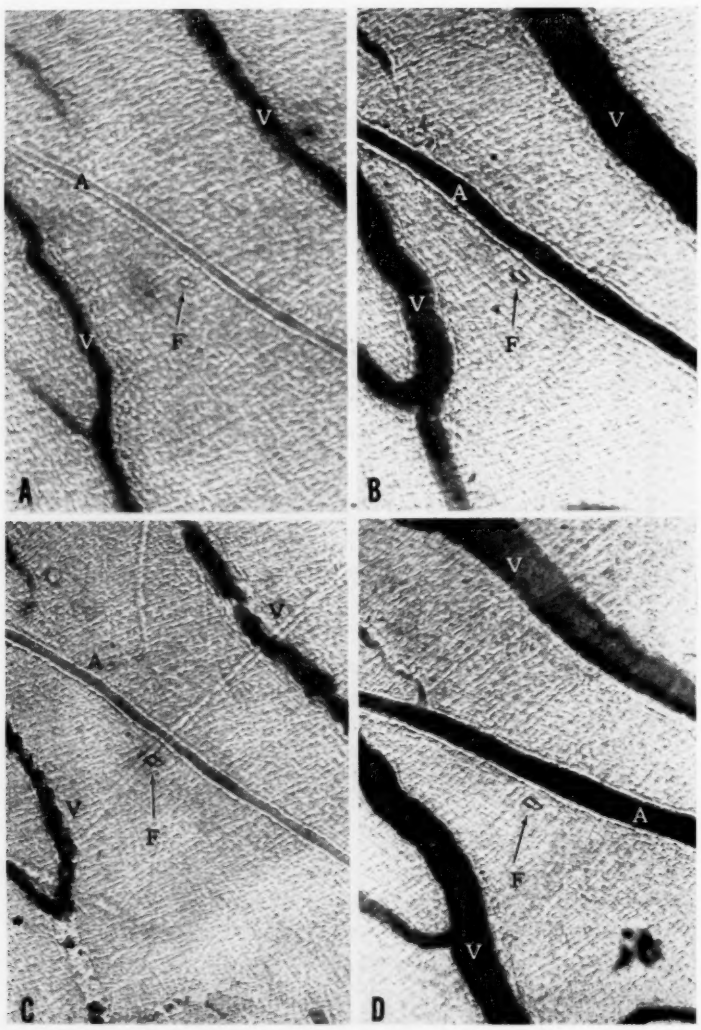

Fig. 5.—*A* = arteriole; *V* = venule; *F* = foreign body.

Figs. 5*A* to 5*H* are photographs of the same area in the same animal at comparable intervals after the second and third injections of horse serum with and without treatment with cortisone. Reference to Fig. 1 (schedule C) will show the schedule of treatment. A small foreign body (*F*) identifies the same arteriolar segment in different photographs. *A*, twelve hours after the second injection of horse serum. Cortisone for nineteen days. Note tone of arteriole (*A*). *B*, eleven hours after the third injection of horse serum. No cortisone for seventeen days. The arteriole (*A*) is widely dilated as are venules (*V*). *C*, four days after the second injection of horse serum. Twenty-third and last day of cortisone treatment. The tone of the arteriole (*A*) and venule (*V*) is well maintained. *D*, four days after the third injection of horse serum. No cortisone for twenty-three days. Arteriole (*A*) and venules (*V*) remain dilated.

Very little arteriolar sticking developed following the second injection of horse serum in treated animals (Figs. 2, 3, and 4). This is in striking contrast to the sticking which developed in the same animals following a third injection of horse serum (Fig. 2*B*), after treatment had been stopped or in untreated controls following the second injection of normal horse serum.[5] Ad-

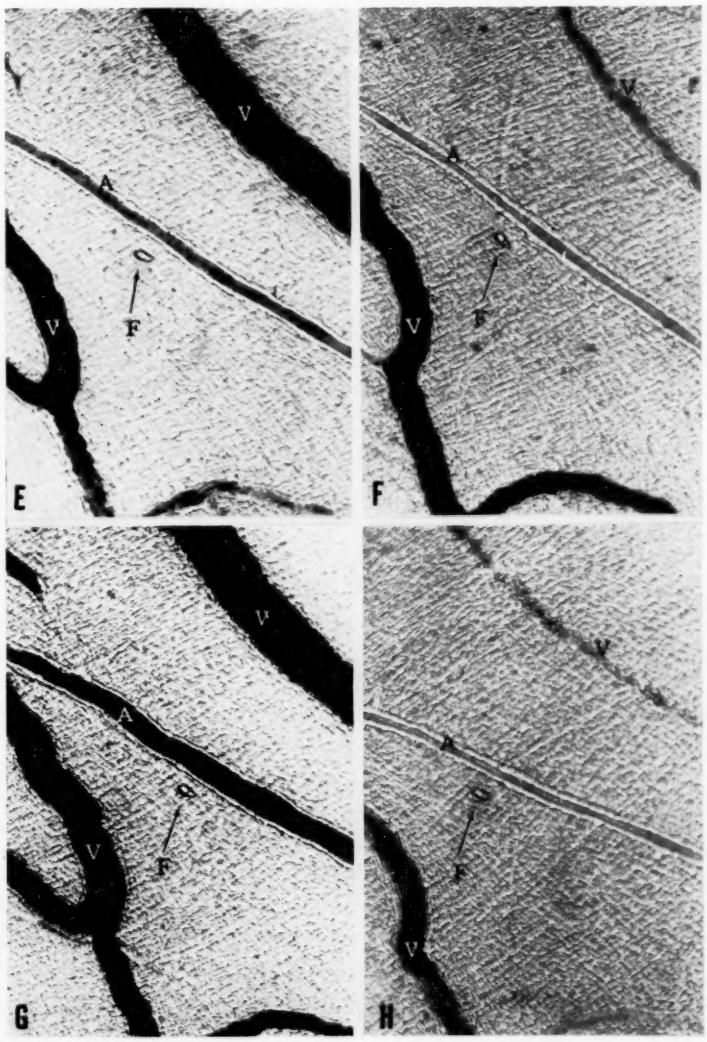

Fig. 5.—*E*, Same arteriole (*A*) and venule (*V*) Fourteen days after the second injection of horse serum. No cortisone for ten days. There has been minimal dilatation of arteriole (*A*) and more marked dilatation of venules (*V*) since stopping cortisone. (×100.) *F*, thirteen days after the third injection of horse serum. A second course of cortisone was started six days after the third injection and this photograph was taken the seventh day of the treatment. Tone of arteriole (*A*) and venule (*V*) has increased again after re-starting cortisone. *G*, twenty-one days after second injection of horse serum. No cortisone for seventeen days. Note dilatation of arteriole (*A*) and venule (*V*). *H*, twenty-one days after the third injection of horse serum. Cortisone for fourteen days (second course of treatment).

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herence of leucocytes to arteriolar endothelium developing after the third injection disappeared when cortisone therapy was resumed (Figs. 2*C*, 2*D*, and 3).

Swelling of arteriolar endothelium was not seen following the second injection of horse serum in any of the cortisone-treated animals. It did develop following cessation of therapy, however, and was frequently seen following the third injection of horse serum.

C. *Changes in venules and capillaries:* Venule and capillary tone was better maintained in treated animals following the second injection of horse serum than in the untreated controls. Loss of tone developed after treatment had been stopped. These changes are illustrated in Fig. 5*A* to 5*G*. Variations in venule and capillary tone seemed to be relatively passive, and reflected change in arteriolar tone.

There also appeared to be significant reduction in the extent and duration of leucocyte sticking to venule endothelium in cortisone treated animals, although this was never completely suppressed. Swelling and proliferation of venule endothelium was not seen during treatment, but sometimes developed after treatment had been stopped, particularly following a third injection of horse serum. Normal vascular endothelium has a refractile appearance, and this was better maintained in treated animals following the second injection of horse serum than in untreated rabbits.

II. *Intravascular Changes.*—

A. *Sludging:* Agglutination of erythrocytes was not suppressed by cortisone as dramatically as were the vascular changes (Fig. 6*D*). If the degree of sludging which developed in treated animals following the second injection of horse serum (Figs. 3 and 4) is compared with that which developed in untreated animals,[5] it is evident that there was a significant reduction. But there was no appreciable difference between the degree of sludging which followed the second injection of horse serum with cortisone treatment and that which followed the third injection without treatment (Figs. 3 and 4). The very marked sludging observed in untreated animals was never seen in the treated animals.

B. *Platelet and leucocyte thrombi and emboli:* Very few platelet and leucocyte thrombi and emboli were seen in treated animals following the second injection of horse serum (Fig. 7) as compared with untreated animals.[5] More were seen following the third injection of horse serum after cortisone had been stopped (Fig. 7), but these differences were not as evident as the differences in vascular response with and without treatment in the same animal.

III. *Exudate.*—

Almost no exudate was seen immediately following the second injection of horse serum in cortisone-treated animals. Exudate did develop following the third injection of horse serum, however. Figs. 6*A* and 6*B* show exudate which developed following the third injection of horse serum in rabbit No. 129. The clearing of this exudate following a second course of cortisone therapy is demonstrated (Fig. 6*C*).

IV. *Systemic Reactions.*—

The second injection of horse serum in the untreated rabbit was often attended by a marked increase in respiratory rate and profuse defecation sometimes accompanied by urination. This usually occurred during the course of the injection.

In the animals treated with cortisone prior to second injection of horse serum these reactions almost completely suppressed. Fig. 8 compares the

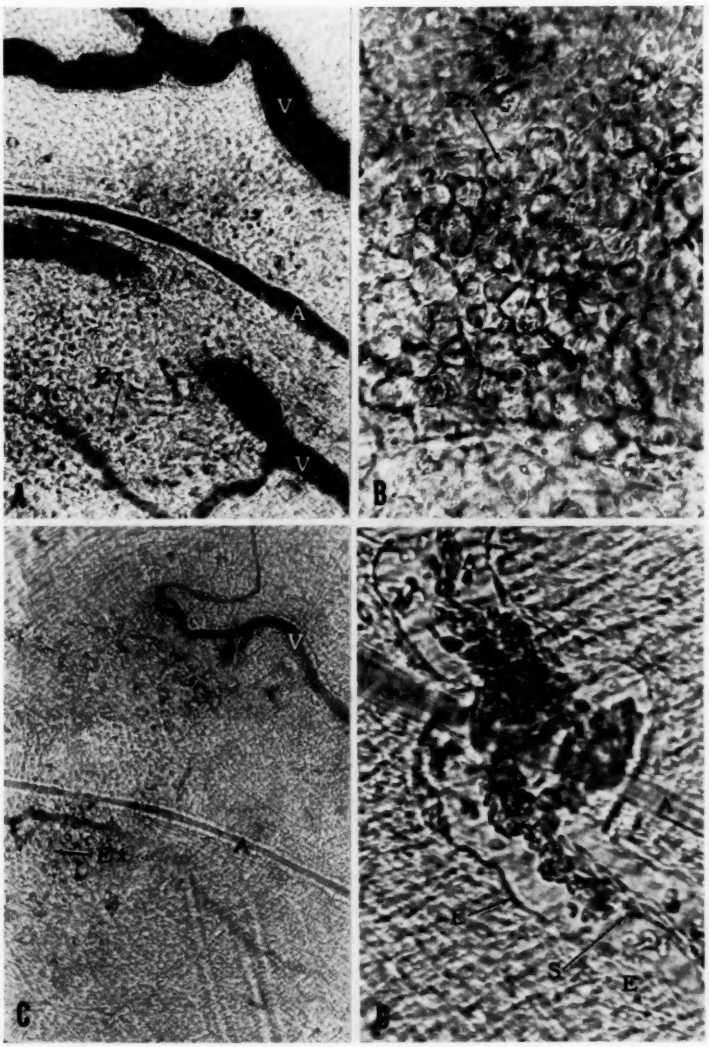

Fig. 6.—*V* = venule; *A* = arteriole; *Ex* = exudate; *S* = sludge; *E* = endothelium. *A*, An area of exudate (*Ex*) adjacent to an arteriole (*A*) and lying over a venule (*V*) partially compressing the venule. This is six days after the third injection of horse serum. No cortisone for twenty-four days. (×100.) *B*, Same area of exudate (*Ex*) under higher magnification. It is made up of large mononuclear cells. (×400.) *C*, The same area after thirteen days of cortisone treatment. Vascular tone has increased and the exudate (*Ex*) has largely disappeared. (×100.) *D*, Sludging (*S*) in venule (*V*) after second injection of horse serum in animal treated with cortisone. (×400.)

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systemic reaction in untreated animals (Group I) with the reaction in treated animals (Group II), and the marked reduction in the reaction as a result of cortisone therapy is apparent. Animals given a third injection of horse serum after cortisone had been stopped had a more marked systemic reaction (Fig. 8, Group III).

DISCUSSION

The present studies present certain facts concerning the effects of cortisone upon the in vivo manifestations of serum disease in rabbits.

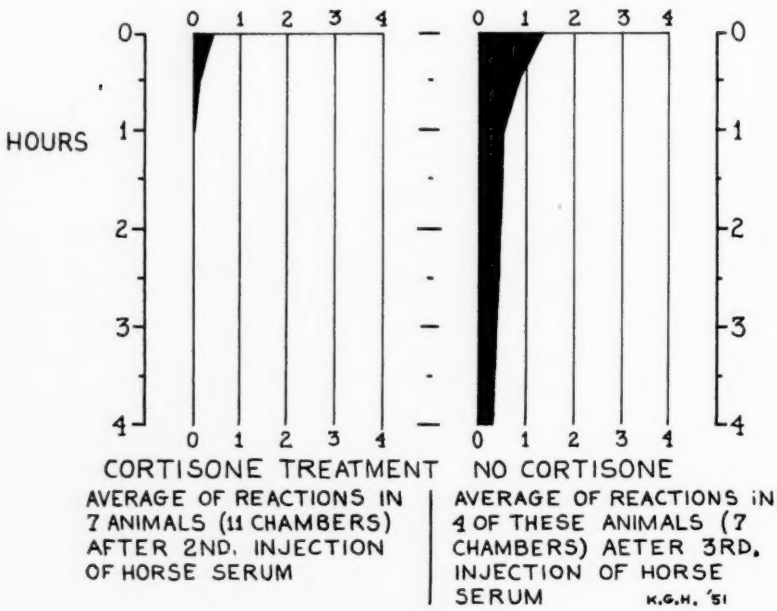

Fig. 7.—Serum sickness. Platelet and white blood cell thrombi and emboli in the same animals with and without cortisone treatment.

First, it is apparent that there is a quantitative reduction in the intensity of the response. The suppression is rarely complete, and in every experiment there is some evidence for the occurrence of the specific reactions. Second, the most profound effect of cortisone is on the vascular reactions. Vascular tone is better maintained, endothelial damage is reduced and, as a result of decreased diapedesis of leucocytes, the amount of exudate is diminished. Third, there is some reduction in sludging and in the occurrence of platelet and white blood cell thrombi and emboli, but the suppression of these manifestations of serum sickness is much less dramatic. Fourth, the modifying effects of cortisone are reversible, and an exacerbation of the vascular changes occurs after treatment has been stopped.

There are several possible ways in which cortisone might suppress the inflammatory reaction in serum sickness. Alteration of antibody formation,

interference with antigen-antibody union, suppression of histamine liberation or activity, and decreased vascular and cellular permeability must all be considered.

At present the role of cortisone in altering antibody formation is in doubt,[6, 7] but there are no data which suggest that the type of immunization procedure employed to produce serum disease (large doses of foreign serum given intravenously) results in any reduction in circulating antibody. Rich and co-workers[1, 2, 3] report that neither cortisone nor ACTH reduced the precipitin response in serum disease, although the vascular and cardiac lesions were suppressed by both lungs.

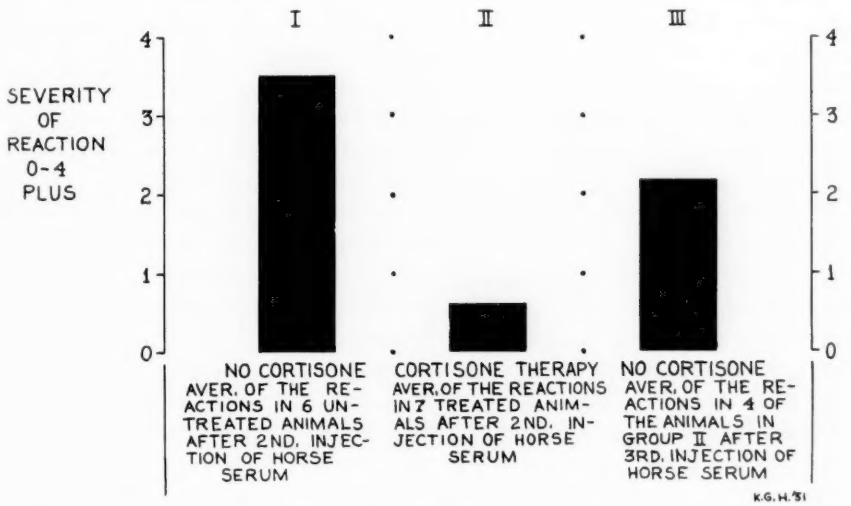
I
II
III
SEVERITY OF REACTION 0-4 PLUS
4
3
2
1
0
NO CORTISONE AVER. OF THE REACTIONS IN 6 UNTREATED ANIMALS AFTER 2ND. INJECTION OF HORSE SERUM
CORTISONE THERAPY AVER. OF THE REACTIONS IN 7 TREATED ANIMALS AFTER 2ND. INJECTION OF HORSE SERUM
NO CORTISONE AVER. OF THE REACTIONS IN 4 OF THE ANIMALS IN GROUP II AFTER 3RD. INJECTION OF HORSE SERUM
K.G.H.'51

Fig. 8.—The effect of cortisone treatment on the systemic reaction in serum sickness.

On the basis of passive immunization experiments Cohen and Moses[8] have suggested that one mechanism by which cortisone may suppress the arteritis of serum disease is by interference with antigen-antibody union. However, Carryer, and Code[9] found that cortisone had no inhibitory effect upon the release of histamine during an in vitro hemolytic reaction in rabbits' blood, and therefore had presumably not suppressed antigen-antibody union.

That cortisone may suppress some of the effects of histamine is suggested by the almost complete absence of the general reactions of defecation, urination, and dyspnea during the second injection of horse serum. It seems likely that these reactions result from smooth muscle contraction. Existing evidence suggests that ACTH, at least, may cause a remarkable decrease in urinary histamine and a rise in urinary histidine in asthmatic patients,[10] but there is no evidence as yet that histamine reactions such as bronchospasm in the guinea pig or wheal formation in the human skin are reduced by these drugs.[11, 12] The factors of dosage and time may not have received sufficient attention in

these studies, especially since the results reported here suggest that cortisone exerts a quantitative rather than qualitative effect. Although it seems likely that histamine metabolism is directly related to adrenal function[13] the precise mechanism by which cortisone and ACTH suppress so many phenomena in which histamine has long been thought to play a role is at present obscure.[7] Whether the changes in arteriolar tone and the preservation of endothelial integrity which have been described in the preceding part of this report may limit the spread of histamine across cellular or vascular membranes remains to be investigated.

It is apparent that the biochemical mechanisms by which cortisone suppresses the vascular and, to a lesser extent, the intravascular reactions in serum sickness are not clear. But the in vivo observations which are recorded here may offer a histologic common denominator for the mode of action of cortisone and/or ACTH in a large number of the inflammatory phenomena. Cortisone and ACTH have been demonstrated to suppress the classical signs of inflammation in a wide variety of acute infections,[14-16] wound healing,[17, 18] the Schwartzman phenomenon,[19] chemical irritations,[20, 21] and thermal burns.[22, 23]

One of the most important components of the acute inflammatory response is reactive hyperemia (rubor). Not only the observations reported here but others in "normal" chambers show that one of the striking effects of cortisone is a maintenance or an increase in arteriolar tone. This might be expected to prevent largely the arteriolar dilatation necessary for reaction hyperemia.

Similarly, in the light of our observations, the formation of edema fluid and exudate (tumor) could be expected to be diminished by cortisone therapy. The endothelium becomes more refractile, endothelial swelling is reduced, and leucocyte sticking followed by diapedesis and exudate formation is greatly inhibited. These in vivo observations suggest that the integrity of the endothelium is protected by cortisone.

They gain more significance when viewed in the light of the important observations of Benditt and co-workers,[24] who have recently reported that the increased permeability of capillaries produced by testicular extract is largely inhibited by ACTH or cortisone therapy.

Other in vivo ear chamber studies in this laboratory have demonstrated that the protective action of cortisone in preserving arteriolar tone and endothelial integrity are demonstrable following trauma, tuberculous infection, and the focal reaction.

It becomes apparent, then, that cortisone or ACTH may have a beneficial or harmful net effect upon an inflammatory reaction depending upon several factors. If the inflammatory stimulus is a bacterial or viral infection, the reduction in active hyperemia and exudation may be deleterious by preventing (a) adequate leucocytic migration for phagocytosis, (b) the dilution effect of edema fluid upon toxic products, and (c) the accumulation of fibrin, antibodies, etc., from the bloodstream in the infected region.

In surgical wound healing the numbers of fibroblast precursors (lymphocytes and monocytes) reaching the injured tissue may be reduced significantly

when the animal is treated with cortisone. Furthermore, during cortisone therapy the absence of hyperemia with its resultant increase in nutriment for cell growth and proliferation may be important in quantitatively retarding would healing. On the other hand, in response to trauma or in the case of thermal burns, these same effects of cortisone upon arterial tone and endothelial integrity may be important in decreasing loss of fluid (preventing shock), decreasing scar formation, etc.

It would appear, therefore, that the effect of cortisone in serum disease is not due primarily to suppression of antibody formation. Cortisone can be said to have a profound effect upon the integrity and reactivity of small blood vessels, and this appears to be capable of causing significant inhibition of the inflammatory response to a variety of stimuli. It should be emphasized that the effect is quantitative and, if an inflammatory stimulus is great enough, a modification of the response may not be apparent.

The effect of cortisone upon vascular reactivity is only one of the many effects of the hormone and may be secondary rather than primary. These observations do help to explain, however, the beneficial effects of cortisone on a wide variety of unrelated diseases and its harmful influence on infection.

SUMMARY AND CONCLUSIONS

Cortisone produces a quantitative reduction in the in vivo manifestations of serum disease in rabbits as observed by the ear chamber technique.

Treatment with cortisone effects a profound reduction in the degree of vascular damage caused by serum disease. Vascular tone is better maintained, vascular endothelium tends to keep its normal refractile appearance, swelling of endothelium is suppressed, and there is reduction in the sticking of leucocytes to endothelium.

Sludging of erythrocytes is less marked, and platelet and white blood cell thrombi and emboli are less numerous in treated animals, but the suppression of these intravascular changes is less dramatic than the reduction of vascular damage.

As a result of the increased integrity of vascular endothelium there is decrease in diapedesis of leucocytes and a reduction in the accumulation of exudate.

There is a reduction in the systemic reaction following the second injection of horse serum in treated animals. The effects of cortisone are reversible, and after treatment is stopped an exacerbation of the vascular changes is observed.

It is felt that cortisone produces a profound effect on the integrity and reactivity of small vessels and that the suppression of the in vivo manifestations of serum disease is due to this nonspecific effect rather than to a suppression of antibody formation or antibody-antigen union.

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IN VIVO OBSERVATIONS OF THE VASCULAR REACTIONS TO LARGE DOSES OF HORSE SERUM USING THE RABBIT EAR CHAMBER TECHNIQUE

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CARDIOVASCULAR lesions in rabbits resembling those of rheumatic fever and periarteritis nodosa in man have been described by Rich and Gregory[1] and others[2-5] in rabbits following large intravenous injections of normal horse serum. In an effort to elucidate the pathogenesis of these lesions an in vivo study has been made of the vascular reactions in serum sickness using the rabbit ear chamber technique. The rabbit ear chamber first described by Sandison[6] provides a thin layer of living vascularized tissue 40 to 50 μ thick. Daily observation of the same tissue can be made in the unanesthetized rabbit under high magnification over a period of weeks or months. Vessels are well differentiated into arterioles, venules, and capillaries. Thus the method is well suited for study of functional change in these small vessels and the supporting connective tissue stroma.

Large intravenous injections of horse serum produce a generalized vascular reaction with lesions in many organs and tissues.[1, 3] This being the case, it seems valid to assume that the small peripheral vessels of the rabbit ear chamber might reflect some of the changes occurring elsewhere. That the rabbit's ear might be a particularly profitable area to observe vascular alteration in serum disease is suggested by the observations of Fleisher and Jones.[7] These workers observed a characteristic erythema and edema of the rabbit's ear following large doses of horse serum.

Serum sickness is accompanied by a consistent vascular reaction in the ear chamber. The nature of this reaction and its relationship to changes in visceral organs is the subject of this report.

METHODS AND MATERIALS

The rabbit ear chamber used in the method of examination has been adequately described elsewhere.[6, 8, 9, 10] Observations are made on mature connective tissue which has grown onto a central table 8 mm. in diameter and 40 to 50 μ thick. Photomicrographs were made with a Leica micro-attachment. The source of illumination was a Bausch & Lomb spherical lamp, and the same lighting sufficed for Kodachrome motion pictures. A water filter was used to avoid heating the tissue.

Observations were made on seven rabbits especially selected for the size of their ears. A large ear is essential for successful insertion of the ear chamber and ease of observation. One animal had chambers in both ears, and the remaining six had one chamber. Body weight varied between 3,100 to 5,400 grams, the average being 4,100 grams.

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This work was sponsored by a grant from the United States Public Health Service.
Received for publication, June 27, 1951.

Sterile untreated horse serum* was used and cultures were made of the horse serum at the time of each injection. No contamination of the horse serum was encountered. The serum was kept under refrigeration and was warmed to 37° C. before injection.

Six animals received two intravenous injections of normal horse serum eighteen to twenty days apart, each dose equaling 10 ml. per kilogram. Using a 50 ml. syringe the horse serum was injected in the marginal ear vein at a rate of 4 ml. per minute. One animal was given an initial injection of normal horse serum followed eighteen days later by normal rabbit serum for control purposes.

The ear chamber was observed during each intravenous injection. Frequent observations, usually daily, were made during the first thirty to forty days of each experiment.

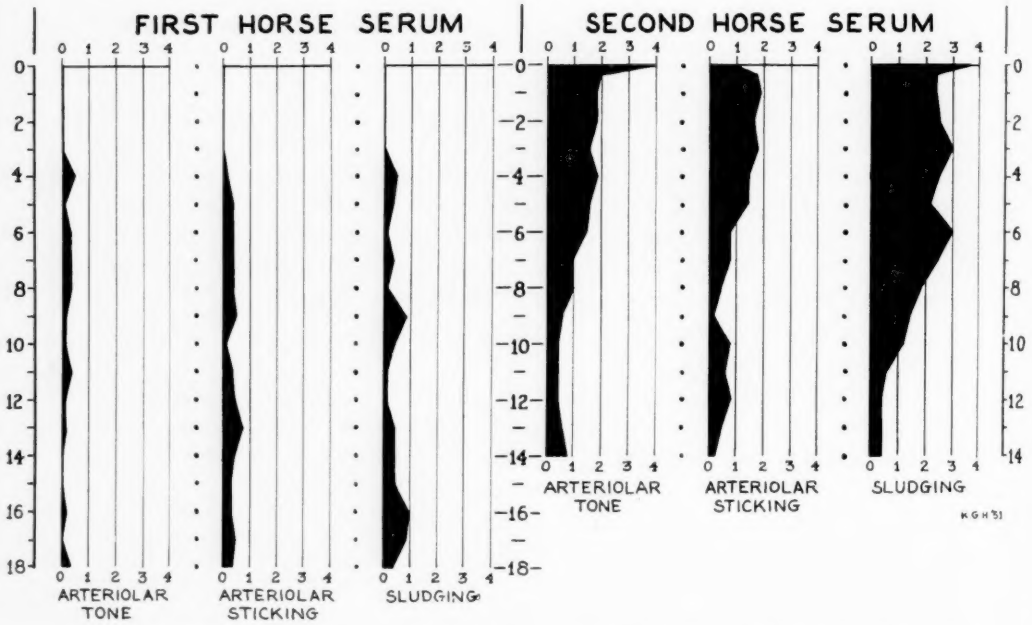

Fig. 1.—Serum sickness. Average of the reactions in six animals after first and second injections of horse serum.

More frequent observations were made during the first twenty-four to seventy-two hours after the second injection of horse serum. Less frequent examinations were made after the thirtieth to fortieth day, but all surviving animals were followed for at least two months.

Evaluation of reactions observed in the chambers was further facilitated by studying movies of each chamber taken prior to injection and at frequent intervals following each injection. These were taken in color and at several magnifications. They made it possible to observe within a few minutes the chronological sequence of the vascular reactions which took days to develop.

One animal died twenty-five hours after the second injection of horse serum, a second four days after the second injection. These were autopsied immediately. The remaining animals were sacrificed and autopsied later. Histopathologic studies were made of all but one animal.

*Generously donated by the Eli Lilly Research Laboratories.

RESULTS

No immediate reaction was observed in the chamber following the first intravenous injection of warm normal horse serum in the rabbit—except for a mild transitory vascular dilatation presumably due to the increase in blood volume. In one animal, the first injection of horse serum was given at icebox temperature by mistake and immediately following injection numerous transient platelet thrombi were seen. Otherwise, platelet thrombi and emboli were not seen after the first injection.

Beginning five to seven days after the first injection, however, certain vascular and intravascular changes were observed. These were minimal and often transitory, but became greatly accentuated following the second injection of horse serum.

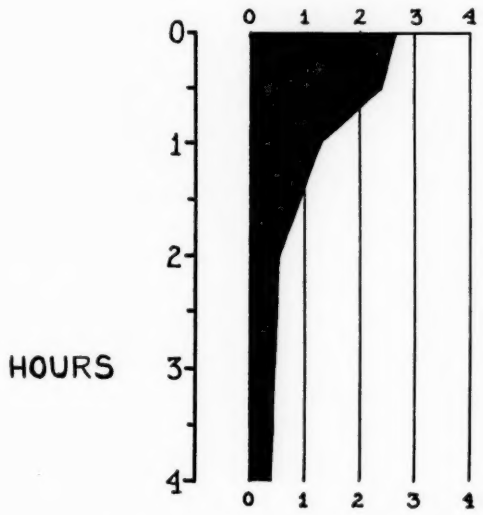

Fig. 2.—Serum sickness. Platelet and white blood cell thrombi and emboli following the second injection of horse serum. Average of the reactions in six animals (seven chambers).

The principal changes were the following:

I. Vascular
   A. Changes in arteriolar tone
   B. Damage to arteriolar endothelium
   C. Damage to venule endothelium

II. Intravascular
   A. Sludging
   B. Platelet and WBC thrombi and emboli

III. Exudate

An attempt has been made to quantitate some of these changes following the first and second injections of horse serum, and the results are shown in Figs. 1 and 2. These figures represent an average of the changes occurring

in the six animals studied (seven chambers) on an arbitrary scale of one plus to four plus. Fig. 1 depicts (1) the changes in arteriolar tone, (2) damage to arteriolar endothelium as evidenced by sticking of white blood cells to arteriolar endothelium, and (3) erythrocyte sludging. In each instance the time relationship to the first and second injections of horse serum is shown. No attempt has been made to quantitate the damage to venule endothelium for reasons which will be discussed below. Fig. 2 shows the frequency of platelet and white blood cell thrombi and emboli following the second injection of horse serum.

In each instance four plus represents the maximum reaction seen in the course of these experiments and one plus represents minimal but definite reaction. The following criteria were used for quantitating the reactions charted: (a) *Arteriolar tone:* Four plus is maximum dilatation and one plus to three plus are various grades of localized constriction and dilatation. (b) *Arteriolar sticking:* Four plus is focal sticking of 25 to 100 leucocytes in short arteriolar segments, and one plus to three plus are various grades of focal sticking between 1 and 25 leucocytes. (c) *Sludging of erythrocytes:* Four plus is agglutination of erythrocytes so marked that the individual contour of red blood cells is lost. One plus is minimal but definite clumping of erythrocytes, and two plus to three plus are intermediate grades of sludging. (d) *Platelet and WBC thrombi and emboli:* Four plus represents numerous clumps of platelets and leucocytes in all larger vessels. One plus to three plus are varying numbers ranging from a few small thrombi and emboli to the maximum.

Figs. 1 and 2 represent the average intensity of these various reactions for six animals.

I. *Vascular Damages.*—

A. *Changes in arteriolar tone:* Beginning four to seven days after the first injection of horse serum, localized constrictions were seen in arterioles 30 to 60 μ in diameter (Fig. 3*A*). These were minimal and transitory, as can be judged from the first column in Fig. 1. Similar changes did not occur in end arterioles.

During and after the second injection of horse serum changes in arteriolar tone were much more striking. In every experiment blood flow stopped on the observation table one and one-half to two minutes after beginning the second injection of horse serum (after 6 to 8 ml. had been injected). This was apparently caused by constriction of larger arteries proximal to the observation table, for the vessels on the table remained filled with stagnant blood, and there was usually progressive dilatation of the visible arterioles. In only one animal was there constriction of the main arteriole on the table occurring thirty seconds after constriction of proximal arteries. The proximal arterial constriction usually lasted seven to nine minutes and then there was slow resumption of flow. During this period and for two and one-half to eighteen hours after the second injection large arterioles were widely dilated. The dilatation was less obvious in small end arterioles.

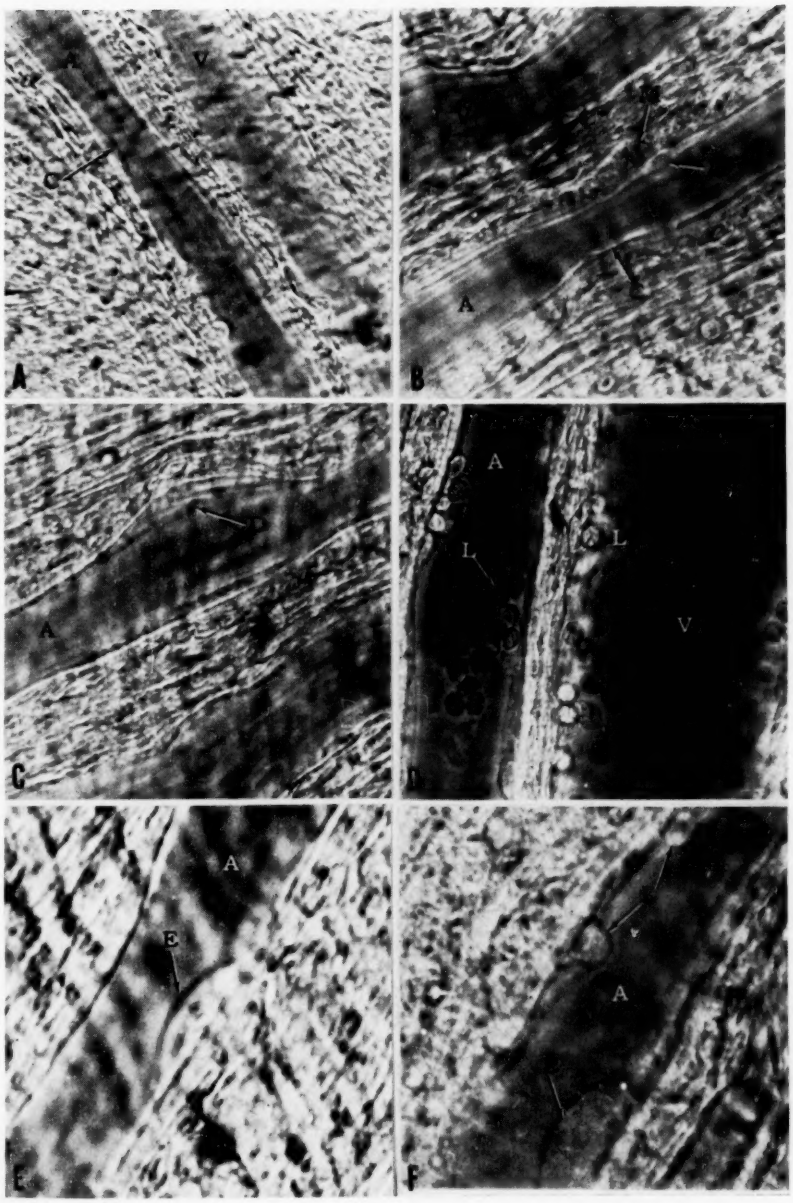

Fig. 3.—*A* = arteriole; *V* = venule; *C* = constriction; *L* = leucocyte; *D* = dilatation; *E* = endothelium; *M* = muscular wall of arteriole.

A, A localized constriction (*C*) in an arteriole (*A*) fourteen days after the first injection of horse serum. A venule (*V*) is adjacent to the arteriole (*A*). (×400.) *B*, a localized constriction (*C*) in an arteriole (*A*) twenty-two hours after the second injection of horse serum. The muscular wall (*M*) of the arteriole is well seen in region of constriction. A leucocyte (*L*) is imbedded in endothelium of the arteriole (*A*). (×400.) *C*, A localized dilatation (*D*) in the wall of an arteriole (*A*) fifty-two hours after the second injection of horse serum. (×400.) *D*, Sticking of leucocytes (*L*) to the endothelium of an arteriole (*A*) and a venule (*V*) twenty-two hours after the second injection of horse serum. (×400.) *E*, Swelling of arteriolar endothelium (*E*) seven days after the second injection of horse serum. (×800.) *F*, Swelling of arteriolar endothelium (*E*) and leucocytes (*L*) adherent to endothelium four days after the second injection of horse serum. (×800.)

As the marked arteriolar dilatation subsided localized constrictions and dilatations appeared (Figs. 3*B* and 3*C*), similar to those seen after the first injection but much more striking. These were usually persistent and intense for the first week following the second injection, and then were seen only intermittently. These changes sometimes persisted for as long as two months after the second injection.

B. *Damage to arteriolar endothelium:* The most striking evidence of arterial endothelial damage was the adherence of white blood cells to the endothelial surface. Such "sticking" was seen in arterioles beginning five to seven days after the first injection (Fig. 3*A*). As can be seen in Fig. 1 sticking of leucocytes became greatly accentuated after the second injection of horse serum.

After this injection, maximum adherence of leucocytes to arteriolar endothelium did not occur immediately but reached a maximum between twelve to twenty-four hours as summarized in Fig. 1 and then gradually subsided. It was much more marked in larger arterioles 30 to 60 μ in diameter than in end arterioles. End arterioles often showed little or no sticking of leucocytes. Sticking was focal in distribution and often 20 to 60 leucocytes could be seen adherent to the endothelium in a relatively short segment of arterioles (Fig. 3*D*). This evidence of focal damage was usually persistent in a given segment of arteriole. Although there was gradual decrease in the extent of arteriolar damage during the week following the second injection, intermittent arteriolar sticking was seen as late as four weeks after the second injection of horse serum.

Other evidence of arteriolar damage was the swelling of arteriolar endothelium usually seen during the first week after the second injection (Figs. 3*E*, 3*F*). Swelling of endothelium occurred in dilated vessels with rapid blood flow and was not similar to the apparent swelling which sometimes occurs as vessels constrict.

C. *Venule endothelium:* It was not possible to quantitate the changes which occurred in venules and capillaries, since minor changes in tone constantly occur and minimal sticking of leucocytes often results from minor trauma. There did appear to be some transitory additional dilatation of venules and capillaries and very mild but generalized leucocyte sticking between the fifth to twelfth day after the first injection of horse serum.

Following the second injection of horse serum there was quite marked dilatation of venules and capillaries which seemed to accompany the change in arteriolar tone. This dilatation gradually subsided over a period of two weeks. During the first two to three days after the second injection, leucocyte sticking was prominent in localized venule segment, and minor sticking was seen for two to three weeks. Very little sticking was ever seen in true capillaries.

Swelling and apparent proliferation of venule endothelium did occasionally occur, however, and was additional evidence that venules were being damaged (Fig. 4*A*).

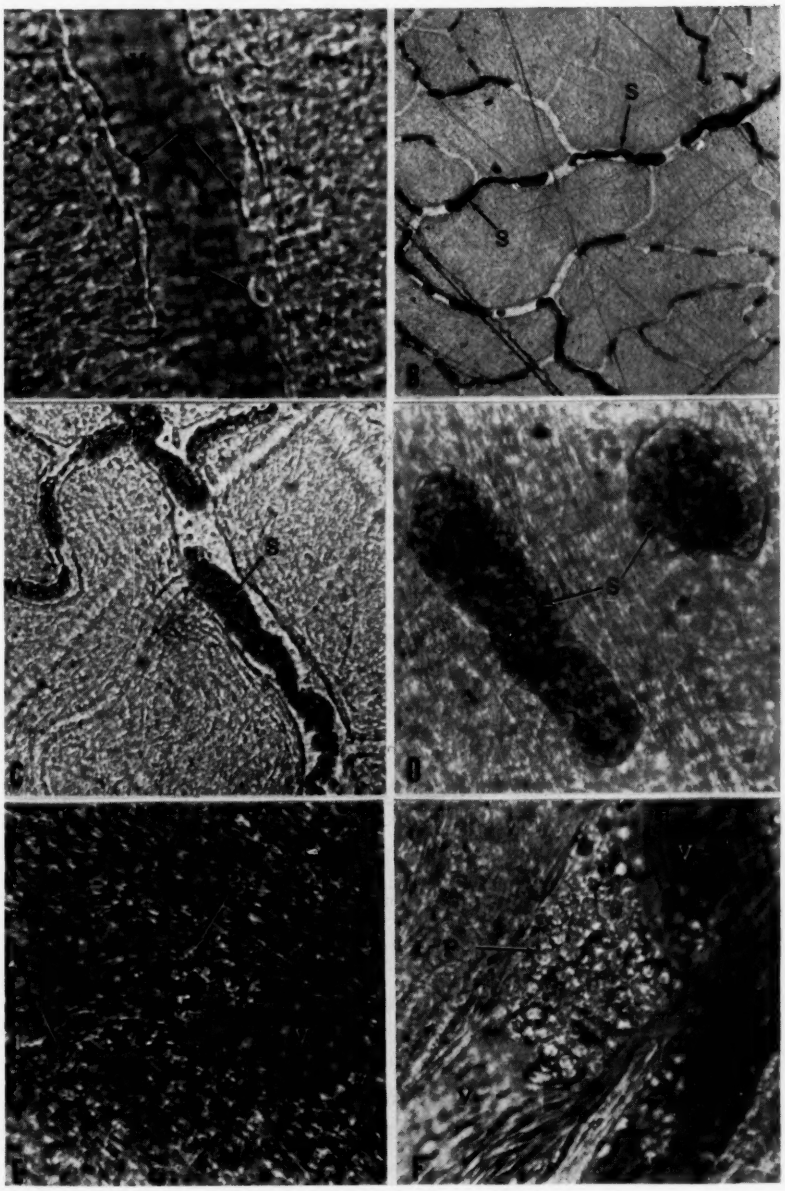

Fig. 4.—*A* = arteriole; *V* = venule; *L* = leucocyte; *S* = sludge; *P* = platelets; *R* = red blood cell; *L-P* = leucocyte and platelet thrombus.

A, Swelling and proliferation of venule endothelium (*E*) and sticking of leucocytes (*L*) in venule (*V*) four days after the second injection of horse serum. (×400.) *B*, Sludging of erythrocytes (*S*) in venules ten minutes after the second injection of horse serum. (×60.) *C*, Higher power of *B* of sludging (*S*) shown in Fig. 4*B*. (×100.) *D*, Sludged erythrocytes (*S*) three days after the second injection of horse serum. This animal died the following day. (×400.) *E*, Platelet thrombus (*P*) in venule (*V*) fifteen minutes after the second injection of horse serum. Red blood cells (*R*) are shown adjacent to platelet thrombus. (×400.) *F*, Leucocyte and platelet thrombus (*L-P*) partially obstructing blood flow in a venule (*V*).

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II. Intravascular.—

A. *Sludging of blood:* The tendency for red blood cells to show intravascular clumping as described by Knisely and co-workers[11, 12] was seen in three of the six animals between the fourth and fifth day after the first injection. In two it was mild and intermittent. In the third it was rather striking and persistent (Fig. 1).

In every animal there was marked agglutination of erythrocytes immediately after the second injection of horse serum, becoming apparent as soon as flow was resumed. There was some decrease after twelve to twenty-four hours, but was followed by a second increase between the third and sixth days (Fig. 1). The degree of sludging was often dramatic, as can be seen in Figs. 4*B*, 4*C*, 4*D*.

B. *Platelet and WBC thrombi and emboli:* A few small clumps of platelets, never more than five to ten platelets in a clump, were seen in only one animal nine to ten days following the first injection of horse serum.

Platelet and leucocyte thrombi and emboli were seen in every chamber as soon as blood flow was re-established after the second injection of horse serum (Figs. 4*E*, 4*F*). Thrombi were seen adherent to arteriolar, venule, and capillary endothelium. The largest thrombi were seen in venules. In all types of vessels they were numerous for the first hour and then rapidly disappeared (Fig. 2). In only two animals were they seen as late as twelve hours after the second injection.

III. Exudate.—

No significant exudate occurred following the first injection of horse serum.

Following the second injection of horse serum diapedesis of white blood cells through both venule and arteriolar endothelium occurred. The amount of exudate was never great, but some degree of periarteriolar inflammatory infiltrate did occur in each chamber. This was not persistent, however, and in no instance was focal necrosis of arteriolar walls nor intense periarteritis seen adjacent to the arterioles of the chamber.

It is important to note that no reaction of any type was observed when normal rabbit serum was substituted for horse serum at the time of the second injection.

Histopathology: Four of the rabbits were sacrificed long after one would expect to see acute vascular lesions. Nevertheless evidence of previous arteritis characterized by fibrosis and disorganization of the media was observed in two of these four animals.

Tissues were examined from only one of the two animals which succumbed shortly after the second injection. This rabbit showed no significant arteritis; the most striking changes being a diffuse subacute myocarditis and acute pulmonary edema and congestion.

DISCUSSION

There can be little doubt that the vascular and intravascular changes observed in these experiments were manifestations of hypersensitivity since they

began four to seven days after the first injection of horse serum at a time when both antigen and antibody are known to be present in the blood stream[13, 14] and were accentuated after the second injection. It is of interest that the time of occurrence of the major portion of the in vivo microscopic vascular reaction corresponds closely with the chronological sequence of gross erythema and edema of the rabbit's ear noted by Fleisher and Jones.[7]

There was no correlation between the severity of the intravascular reaction (sludging and platelet and white blood cell thrombi and emboli), and the intensity of the vascular response (constriction and dilatation of arterioles and endothelial swelling). In fact, sludging was most striking in the three animals which showed the least vascular reaction. The chambers in these three animals were rather thin, and the arterioles were all small. The rate of blood flow is usually slower in thin chambers, and this may have accounted in part for the degree of sludging seen.

These experiments failed to demonstrate in vivo the necrotizing arteritis described by various investigators as occurring in visceral organs[1-5] but they did show that even in peripheral vessels focal endothelial damage and change in vascular tone were consistent findings. It should be remembered that the vessels observed in the rabbit ear chamber were smaller than those in which periarteritis is usually seen. Rich and Gregory[1] suggested that the damage to the media of arteries in experimentally produced periarteritis nodosa might be the result of the damage to the endothelium of the intima, and the present observations demonstrate that endothelial damage is a prominent part of the vascular reaction. It seems likely that the damage to vascular endothelium is generalized (though focal in distribution) and it may well be a precursor of the arteritis found at autopsy in heart, kidney, lungs, and other viscera.

For the most part, the endothelial damage to arterioles described in this paper was transitory. Hawn and Janeway[4] have described reversible vascular changes in the kidneys of rabbits sacrificed at various intervals, after the injection of foreign protein. They also remarked that the arterial lesions seemed reparable within a short time.

Spasm of arteries and arterioles in response to anaphylaxis has been observed repeatedly.[15, 16] In the present experiment it is of interest to note the variable response of different sized vessels, and to speculate on what part change in tone may play in the pathogenesis of the arteritis of serum sickness.

Although spasm of arteries supplying the tissue of the chamber apparently occurring during the second injection of horse serum, almost complete loss of tone seemed to be the characteristic response of the visible arterioles in the chamber immediately after this second injection. Tone was gradually regained, but localized dilatations and constrictions (Figs. 3*B* and 3*C*) persisted. These focal changes were variable in extent from day to day but tended to persist in the same segment of arteriole, and the most marked sticking of leucocytes to endothelium usually occurred in the same regions. Hopps and

Wissler[2] suggested that vascular spasm might provide a *locus minoris resistentiae* responsible for localizing the inflammatory reaction in focal regions of small and medium sized arteries. The present experiments suggest that endothelial damage is most marked in the region of localized constrictions and dilatations. End arterioles with only scattered muscle cells did not show similar changes in tone, nor was endothelial damage ever as great as in larger arterioles.

It is possible that even more marked focal spasm and dilatation occurs in arteries with better developed muscular walls. The focal character of the arterial constrictions and the relatively mild involvement of the arterioles may help explain the failure of rabbits with serum disease to develop hypertension.[17]

Little attention has been paid to the possible role of intravascular changes in the pathogenesis of serum sickness. More and Waugh[18] found a decrease in the coagulation time of rabbits during the first seven days following massive injection of bovine serum gamma globulin, and postulated that fibrin thrombin might form in glomerular capillaries and contribute to the development of glomerulitis.

It is possible that platelet thrombi and agglutinated masses of red blood cells may cause occlusion of small vessels particularly in lung and kidney. Such thrombi are characteristic of the Arthrus reaction as observed in vivo[19] and, although thrombosis has not been observed in these experiments, the basic response in serum sickness and the Arthrus reaction is the same. Possibly the capillary occlusion in rheumatic pneumonitis and sulfonamide sensitivity described by Rich and Gregory[20] is caused by this mechanism.

Histamine may also be a factor in the endothelial damage. Rabbit platelets are known to contain histamine,[21] and there is disintegration of platelets following their agglutination. Furthermore, a marked fall in the platelet count can be demonstrated following the second injection of horse serum (unpublished observations).

These observations suggest that both endothelial damage and changes in the arterial media may be of importance in initiating the arteritis accompanying serum sickness in rabbits. They do not enable one to decide whether one of these is of primary importance and leads to the other. They do show that several important vascular and intravascular phenomena occur regularly in vivo following injection of horse serum, and that these responses occur at the same time that serum sickness is likely to develop. Thus they serve admirably as a basis for study of the modes of alteration of the process of certain drugs. This is the subject of the following report.

SUMMARY AND CONCLUSIONS

Using the rabbit ear chamber technique on in vivo study has been made of the vascular reactions following large intravenous injections of normal horse serum eighteen to twenty days apart.

Four to seven days after the first injection localized constrictions and dilatations were seen in the walls of arterioles 30 to 60 μ in diameter, and there was segmental sticking of leucocytes to arteriolar endothelium. These changes were greatly intensified after the second injection. Often swelling of arteriolar endothelium was seen after the second injection. Smaller arterioles showed minimal changes.

Focal swelling and proliferation of venule endothelium was seen after the second injection. Minimal venule sticking and dilatation occurred simultaneously with arteriolar changes.

Minimal sludging of blood frequently developed four to seven days after the first injection, always becoming intensified immediately after the second, usually with a subsequent increase two to six days later.

Platelet and white blood cell thrombi and emboli were seen during and immediately after the second injection. These were numerous for several hours but were rarely seen after twelve hours.

Normal rabbit serum given nineteen days after horse serum produced no significant change.

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STRESS STUDIES ON AUTO RACE DRIVERS

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NUMEROUS investigators have shown that experimental animals under stress show evidence of increased adrenocortical activity. Studies on human beings subjected to various forms of physical and mental stresses are not so numerous. Stein and associates[1] showed that human beings during environmental and exercise stress showed evidence of adrenocortical activity as measured by a decrease in circulating eosinophils.

We have attempted to evaluate the effect of combined physical and mental stress on normal young healthy males. The "stress" in this study was the annual 500 mile Indianapolis Speedway Race (1950). This is a competitive and dangerous contest in which the drivers race their cars around a 2.5 mile oval track at average speeds of between 120 and 125 miles per hour. This race was halted because of rain after the winner, J. P., had covered 345 miles at an average speed of 124.002 miles per hour. Although the physical stress was limited to this period, the psychological stress was undoubtedly of longer duration. For several days prior to the contest, the subjects were exposed to a constantly increasing tension. Worry about the mechanical condition of their vehicles, attention by the press, and a keen desire to share in the prize money were contributory factors. Several of the subjects could not relax enough to sleep the night before the race.

METHODS AND STUDIES

All drivers were between 20 and 35 years of age. Control studies on all subjects were carried out at least one week prior to the race. The same procedures were carried out within one hour after completion of the race or withdrawal of the driver from the contest.

Eosinophil counts were done by the method of Randolph.[2] Urinary and serum sodium and potassium were measured by the method of Mosher,[3] using a modified Beckman flame photometer. Urinary 17-ketosteroid excretion was determined by the method of Holtorff and Koch.[4] Urinary uric acid was measured by the method of Buchanan and associates,[5] creatinine by the method of Bonsnes and Taussky,[6] and chloride by a modification of the method of Harvey.[7]

RESULTS

Eosinophil Response.—Of the nine subjects in which eosinophil counts were obtained during the control period and immediately after the race, seven subjects had a 90 per cent or greater decrease in the number of circulating eosinophils. Two of the subjects had a decrease of 62 and 65 per cent, respectively, but one of these individuals was forced to discontinue the race after only twenty-five laps. The eosinophil response is charted in Table I.

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Received for publication, June 1, 1951.

TABLE I. EOSINOPHIL COUNTS

DRIVER	CONTROL COUNT (PER CU. MM.)	POST RACE COUNT (PER CU. MM.)	PERCENTAGE DECREASE (PER CENT)
J. McG.	55	0	100
C. G.	140	8	94.7
J. R.	140	49	65
D. R.	173	66	62
J. F.	271	19	93
L. W.	381	22	94.3
J. P.	235	15	93.6
D. C.	469	19	95.9
T. B.	436	7	98.6

17-Ketosteroid Excretion.—In six of the seven subjects in which 17-ketosteroids could be determined during the control and stress periods, there was an increase in excretion of approximately 50 per cent during the period of stress. In one subject there was no change in 17-ketosteroid excretion. The results of the 17-ketosteroid studies are shown graphically in Table II.

TABLE II. DATA ON URINARY 17-KETOSTEROID EXCRETION

DRIVER	PERIOD	MG. 17-KETO-STEROID (PER 100 ML.)	COLLECTION TIME (HR.)	URINE VOLUME (ML.)	TOTAL 17-KETO-STEROID (MG.)	MG. 17-KETO-STEROID (PER HR.)
J. McG.	Control	1.6	11.5	275	4.4	0.38
	Race	3.0	5.0	128	3.8	0.76
C. G.	Control	2.2	13.0	218	4.7	0.46
	Race	2.2	5.0	102	2.3	0.36
J. R.	Control	1.8	4.5	196	3.5	0.77
	Race	5.1	3.0	79	4.0	1.33
D. R.	Control	0.8	10.5	480	3.9	0.37
	Race	2.9	2.5	60	1.8	0.72
J. F.	Control	0.6	12.5	680	4.1	0.33
	Race	1.5	5.0	175	2.5	0.50
J. P.	Control	1.4	9.0	214	3.0	0.33
	Race	4.2	5.5	114	4.8	0.87
D. C.	Control	2.3	3.0	140	3.2	1.06
	Race	4.7	4.0	120	5.6	1.47

Serum and Urine Electrolyte Studies.—The sodium and potassium content of the serum and urine was determined. In addition, the urine was examined for uric acid and creatinine. Analysis of the data failed to show any definite pattern of response. This could have been due to the loss of electrolytes in the unknown volume of sweat, coupled with the impossibility of controlling the diet of the subjects.

DISCUSSION

Rud[8] has shown that there may be a variation in the eosinophil count in normal individuals, but in none of the persons studied by this investigator was the variation over 68 per cent. Since seven of the nine subjects in this study showed greater than 90 per cent decrease in the post-race count as compared to the control, we believe that this is evidence of increased adrenal cortical activity in response to the physical and mental stress of this competitive sport.

The exact significance of the increase in 17-ketosteroid excretion is difficult to evaluate. Whether the observed excretion was expressed as milligrams per liter, milligrams per hour, or as any combination of these parameters, a greater quantity of ketosteroid material was excreted during the race than before (see Table II). The method employed in this laboratory ordinarily gives values of from 12 to 17 mg. per twenty-four hours for normal males of comparable age. It is of interest, however, that this increased 17-ketosteroid excretion during the race period was 50 per cent greater than the amount excreted during the control period in six of seven of the subjects tested.

SUMMARY

Seven of nine subjects showed at least a 90 per cent decrease, and two subjects a 62 and 65 per cent decrease in the total number of circulating eosinophils after being subjected to the physical and mental stress of the Indianapolis 500 mile Speedway Race.

Six of seven subjects tested showed an increased excretion of 17-ketosteroids of at least 50 per cent following the stress as compared to the control period.

There was no consistent response in the sodium, potassium, uric acid, or creatinine in studies that were done on urine and serum.

CONCLUSIONS

Race drivers who were subjected to the physical and mental stress encountered in participation in the 500 mile Indianapolis Speedway Race showed evidence of increased pituitary-adrenal stimulation during the stress period.

The technical assistance of Miss Helen Noble, Mr. Ralph Carmichael, and Mr. O. A. Harvey is gratefully acknowledged.

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ELECTROPHORETIC STUDIES ON THE SERUM OF RHEUMATOID ARTHRITIS PATIENTS TREATED WITH CORTISONE

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ELECTROPHORETIC analyses of sera of patients with rheumatoid arthritis have often indicated characteristic differences from normal with respect to the relative distribution of the major protein components.[1-5] The usual findings have been a relative decrease in albumin level and a relative increase in level of alpha and gamma globulins. That these changes in protein level are not specific is well known.[6, 7] Dole and Rothbard,[5] in a careful study, analyzed serum proteins of one rheumatoid arthritis patient over a period of more than three hundred days. In addition to changes in levels of albumin, and alpha and gamma globulins, they noted that beta globulins were on the lower limit of normal values, and observed random fluctuations in total protein concentrations. Clinical recovery in their patient was associated with return to normal levels.

In view of the clinical response of rheumatoid arthritis patients to administration of cortisone,[8] it was of interest to determine the effect of cortisone therapy on serum abnormalities. A series of serum samples secured from three patients with rheumatoid arthritis prior to and during treatment with cortisone has been studied.

EXPERIMENTAL

The serum was obtained from blood samples taken prior to the daily administration of cortisone. These samples were allowed to clot and to stand at room temperature for several hours prior to centrifugation. To one part of serum was added three parts of veronal buffer at a pH of 8.6 and ionic strength (μ) of 0.10. The specific conductivity of the buffer was $K = 0.00302$ ohms^{-1} cm.$^{-1}$. The system was then dialyzed for forty-eight hours at approximately 2° C. against three changes of the same buffer. The samples were run in a conventional Tiselius electrophoresis assembly equipped with Philpot-Svensson schlieren optics.[9, 10] The system was subjected to a potential gradient of approximately 6 volts cm.$^{-1}$ for one hundred fifty minutes in an all glass cell assembly at a temperature of 0.4° C. Protein composition was determined from tracings of enlargements from photographs of the descending pattern in the usual manner.[11] As predicted by theory[12, 18] and demonstrated experimentally by Armstrong, Budka, and Morrison,[16] errors resulting from the superimposed gradients of both buffer and protein ions are less in the descending pattern. The customary assumption was made with regard to the refractive indices of the individual components.[13]

Little information is available concerning the fluctuations in the levels of various serum protein components with time in a single subject; however Dole,[17] observing the serum pattern of one normal subject over a period of one hundred twenty-five days, noticed random variations in the relative levels of the major components.

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Supported in part by Grants from the Chicago Heart Association and the U. S. Public Health Service.

Received for publication, May 21, 1951.

Normal values for the weight fraction of the individual serum components were taken as follows: albumins 0.601, alpha one globulins 0.052, alpha two globulins 0.095, beta globulins 0.134, gamma globulins 0.118. These values represent the averages reported from several laboratories, as well as our own, using the buffer system employed by us.[14-17] It is well known that electrophoretic analysis at 2 per cent protein concentration in $\mu = 0.10$ buffer systems does not give the correct values for relative amounts of the various components.[15, 16] Nevertheless, for comparison purposes such computations are valid so long as the same buffer system and protein concentration is employed throughout the entire study. We have used the value 1.50 for the normal ratio of albumins to globulins. This value is consistent with the values reported by other workers.[14, 17]

A series of samples of serum from each patient taken at intervals of several days was analyzed. Samples were taken prior to and subsequent to, the initiation of daily cortisone injections.

Total protein concentrations were not determined since ample evidence[1-5, 17, 21] indicates that while random variations occur in total protein levels, the sera of rheumatoid arthritis patients do not differ from those of normal individuals with respect to this factor.

CLINICAL DATA

Patient H. S. was a man, aged 31 years, who complained of painful stiffness in the knees, right shoulder, and hands for eight months. General physical examination was negative, excepting for the joints. The left knee was swollen, contained excess fluid, and was tender on pressure. Metatarsophalangeal joints of both feet were swollen and tender. Joints of the fingers and both shoulder joints were tender but not swollen. Urinalysis showed no albumin or sugar. No abnormal elements were found in the sediment. Level of hemoglobin was 15.3 Gm., erythrocyte count, 5,250,000, leucocyte count, 6,000, and sedimentation rate of the erythrocytes was 10 mm. in one hour (Westergren). Roentgenograms of the left knee showed soft tissue swelling; no osseous destruction. The diagnosis was rheumatoid arthritis. Preliminary treatment consisted of physical therapy, regulated rest, and high-calorie, high-vitamin, anti-constipation type of diet.

In addition, he received injections of gold in the form of gold sodium thioglucose (Solganol B-Schering). Injections were given twice weekly for three months. Total amount of gold compound administered during this period was 250 mg. During this period, the patient showed no loss of weight, and no untoward or toxic effects were noted as a result of the administration of gold. Administration of cortisone was begun March 22, 1950, and continued for twenty-seven days. During this period, he received 2.150 Gm. Daily injections usually consisted of 100 mg. of cortisone. Symptoms improved progressively during this period.

Patient A. B. was a woman, aged 50 years. She complained of painful swellings of the knees, elbows, and shoulders. These symptoms had been present for six months. She noted marked weakness, inability to rise from a chair without help, and complained of severe pain in the affected joints. Stiffness was marked in the morning on arising and improved with activity. She had received no prior treatment for the arthritis.

Physical examination was essentially negative, except for findings referable to the joints. Elbows and wrists were swollen and tender. Metacarpophalangeal joints and the row of interphalangeal joints were swollen, limited in range of motion, and tender. Metatarsophalangeal joints were swollen and tender. Laboratory data were as follows: urinalysis negative, level of hemoglobin, 11.9 Gm., erythrocyte count, 3,850,000, leucocyte count, 6900, and sedimentation rate of erythrocytes was 23 mm. in one hour (Westergren). This patient had lost no weight.

Beginning on March 22, 1950, she was treated with cortisone over a period of thirty days. During this time, she received 1.975 Gm. Usual daily doses consisted of 100 mg., with the exception of a period of eleven days commencing on the sixteenth day of treatment, during which period she received 0.275 Gm.

Symptoms improved progressively throughout the month of treatment. During this time, her diet consisted of usual foods, including meat daily, vegetables, fruit, milk, etc.

Patient E. M. was a woman, aged 36 years. She complained of painful swellings of multiple joints over a period of ten years. Physical examination was essentially negative, excepting for finds referable to the joints. The left elbow was held in a position of flexion deformity, and was swollen and tender. Wrists were swollen and greatly limited in motion and tender. Hands showed characteristic deformity of rheumatoid arthritis, with ulnar deviation of the fingers. Metacarpophalangeal joints were bilaterally swollen and tender, and she was unable to close the fists. The knees were greatly swollen and contained excess fluid and were tender. Laboratory data were as follows: urinalysis was entirely negative. Hemoglobin level was 11.5 Gm. per cent, erythrocyte count, 3,650,000, leucocyte count, 9,000, and sedimentation rate of erythrocytes was 32 mm. in one hour (Westergren).

This patient had received no treatment other than physical therapy at any time prior to this examination. She was given cortisone over a period of twenty-seven days, the total amount being 1.70 Gm. During the first sixteen days, 1.4 Gm. were administered, the dosage being 100 mg. six days weekly. During the subsequent eleven days, 300 mg. were given in amounts ranging from 25 to 100 mg. daily.

Symptoms of arthritis improved progressively during this period of treatment.

RESULTS

The results are given in Table I.

The cumulative dosages of cortisone that each patient received prior to the date the particular serum sample was obtained are shown in the last column of the table. A twenty-four hour period elapsed between the administration of cortisone and the withdrawal of a blood sample for analysis. Fig. 1 shows the findings in a graphic manner. In this figure we have plotted the ratio of the experimentally observed value to the "average" value for clinically "normal" individuals. In both the table and the figure the range of normal values has been taken as twice the standard deviation on either side of the average value. According to statistical methods this range would include 96 per cent of so-called "normal" individuals. These ranges are shown at the ordinate in the figure. Although the serum patterns of all three patients differ from the average levels for normal subjects, only one patient, E. M., displays strikingly abnormal values. In the case of E. M. the levels of both the alpha one and alpha two globulins are elevated well beyond normal limits; while the beta and gamma globulin levels are within normal limits. The albumin level was close to the lower limit of the normal range. In the case of this patient it is of interest that there occurred a general decrease in the levels of the alpha one globulins immediately after the initiation of cortisone treatment. The marked increase in the levels of alpha one, and beta globulins in the final sample of patient E. M. analyzed is of particular interest, since it was associated with a corresponding increase in the erythrocyte sedimentation rate.

Patient A. B. prior to cortisone treatment differed from E. M. in that the alpha one and alpha two and gamma globulins were within normal limits, while the beta globulins were above and the albumin below normal limits. After cortisone treatment was commenced no significant change occurred in the albumin or in the beta and gamma globulin levels; whereas an elevation occurred in the alpha one and alpha two globulins sufficient to raise the levels above normal limits. This rise was likewise associated with an increased erythrocyte sedimentation rate.

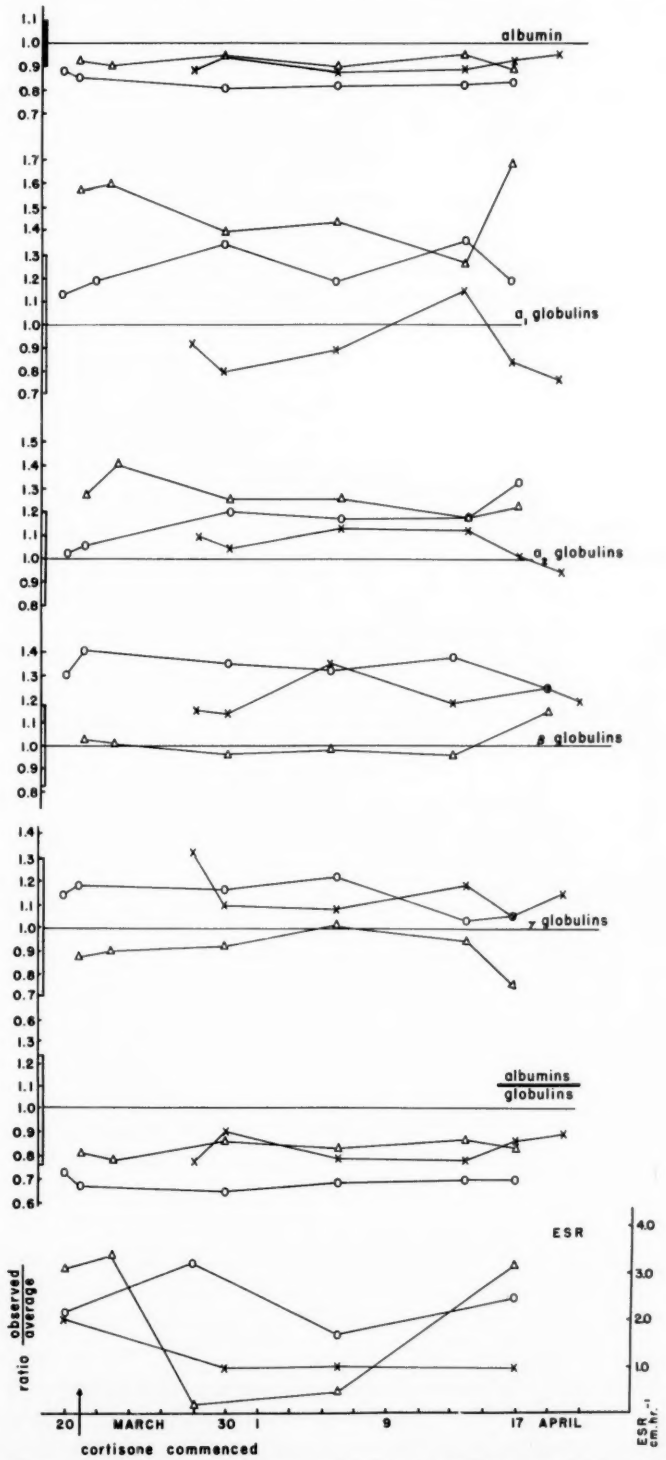

Fig. 1.—Serum protein levels in three rheumatoid arthritis subjects. Ordinate is the ratio of the observed to normal level. Abscissa shows date sample was obtained. Normal range of values shown on ordinate. Erythrocyte sedimentation rate on bottom frame. △ = subject E. M.; O = subject A. B.; X = subject H. S.

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TABLE I. SERUM PROTEIN LEVELS EXPRESSED AS PER CENT OF TOTAL PROTEIN

PATIENT AND SAMPLE	DATE SAMPLE WAS TAKEN	ALBUMINS	α_1 GLOBULINS	α_2 GLOBULINS	β GLOBULINS	γ GLOBULINS	RATIO A/G	ESR CM./HR	CUMULATIVE TOTAL OF CORTISONE ADMINISTERED IN MG.*
ACCEPTED NORMAL VALUES (14-17)		% PROTEIN COMPOSITION							
		60.1 ± 6.0	5.2 ± 1.5	9.5 ± 2.0	13.4 ± 2.4	11.8 ± 3.5	1.50 ± 0.36	1.0	None
EM-1	3-21-50	55.6	8.2	12.1	13.8	10.4	1.25	3.2	None
2	3-23-50	54.1	8.4	13.6	13.5	10.6	1.18	3.4	50
3	3-28-50	--	--	--	--	--	--	0.1	600
4	3-30-50	57.1	7.4	12.0	12.7	10.8	1.33	--	800
5	4- 6-50	55.5	7.5	12.0	13.0	11.8	1.25	--	1400
6	4-10-50	--	--	--	--	--	--	0.4	1400
7	4-14-50	58.3	6.6	11.5	12.6	11.2	1.40	--	1600
8	4-17-50	55.6	8.8	11.8	15.4	8.6	1.23	3.2	1700
AB-1	3-20-50	52.9	5.9	9.7	18.1	13.6	1.12	2.3	None
2	3-21-50	51.1	6.2	10.0	18.8	13.9	1.03	--	None
3	3-28-50	--	--	--	--	--	--	3.3	600
4	3-30-50	49.8	7.0	11.5	18.1	13.7	0.99	--	800
5	4- 6-50	50.8	6.2	11.2	17.6	14.3	1.03	1.7	1400
6	4-14-50	51.1	7.1	11.3	18.4	12.2	1.05	--	1575
7	4-17-50	51.6	6.2	12.8	16.8	12.4	1.07	2.5	1675
HS-1	3-20-50	--	--	--	--	--	--	0.2	None
2	3-23-50	53.5	4.8	10.4	15.4	15.7	1.15	--	100
3	3-28-50	57.7	4.1	10.0	15.3	12.9	1.36	--	425
4	3-30-50	54.1	4.6	10.8	17.8	12.6	1.18	1.0	725
5	4- 6-50	54.1	5.7	10.8	15.7	13.9	1.18	1.0	1250
6	4-14-50	56.5	4.4	9.7	16.7	12.4	1.30	--	1950
7	4-17-50	57.7	4.0	9.0	15.9	13.4	1.36	1.0	2150

*Values in this column refer to amount cortisone administered up to and including period twenty-four hours prior to the date shown in left column.

We were unable to obtain an analysis of the serum of H. S. prior to cortisone treatment. In the case of this patient the levels of the various serum components were essentially normal except for a rather high level of beta globulins in one sample.

Treatment with cortisone produced no apparent change in the relative levels of albumin, beta and gamma globulins, nor in the albumin to globulin ratio in any of the three patients studied. No consistent changes in the levels of alpha two globulins occurred during cortisone treatment in patients E. M. and H. S. In patient A. B. the alpha two globulins were somewhat elevated during cortisone treatment as compared to pre-cortisone patterns. Aside from random fluctuations, the alpha one globulins remained unaffected in the case of A. B. and H. S., whereas in E. M. a progressive drop in the level of alpha one globulins commenced with daily cortisone injections. A subsequent increase in alpha one globulins occurred coincident with an increased erythrocyte sedimentation rate. In the case of A. B. and E. M. the changes in alpha one globulins and the erythrocyte sedimentation rate followed a similar course.

It seems significant that this rise in the erythrocyte sedimentation rate and the increase in alpha one globulins occurred between the sixteenth and the twenty-seventh day of treatment. An explanation for this occurrence may be that these patients (A. B. and E. M.) received only 300 mg. of cortisone during the eleven-day interval.

DISCUSSION

The sera of three patients gave three different electrophoretic patterns prior to and throughout cortisone treatment.

The subjects of this study were diagnosed rheumatoid arthritis; this diagnosis was in each instance established on generally accepted clinical criteria for the disease. The subjects were chosen at random and without prior knowledge of sedimentation rates or serum protein abnormalities. One subject, H. S., was studied even though the sedimentation rates were normal. This occurrence is not unusual. Had we studied a larger number of cases, we might have encountered some with more pronounced abnormalities in their serum protein levels. Our results indicate, however, that pronounced deviations from normal are not always present and that clinical evidences of improvement associated with administration of cortisone are not necessarily accompanied by notable effects on serum protein patterns.

These results differ from those of Olhagen[19] who reported profound changes in plasma components in rheumatoid arthritis treated with cortisone, and are more in accord with the observations of Ropes[20] and Brown.[21] Ropes noted changes in the electrophoretic pattern of one patient and no apparent changes in the pattern of another patient after cortisone treatment. In the first instance the protein pattern was strikingly abnormal; whereas in the second patient the pattern was more nearly normal prior to treatment. Brown and co-workers studied four rheumatoid arthritis cases treated with cortisone. Electrophoretic analysis of the serum patterns obtained before and after corti-

sone treatment indicate, that within the experimental error of the method, in three of the four patients no changes in the serum protein levels occurred during the period of cortisone therapy.

SUMMARY

Random fluctuations in the levels of alpha one, alpha two, and beta globulins were noted in the serum of three patients with rheumatoid arthritis treated with cortisone. The favorable clinical response to cortisone was not accompanied by a return of albumin and alpha one globulins to normal values during the period of cortisone therapy.

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THE EFFECTS OF ACTH AND CORTISONE ON THE RENAL TUBULAR TRANSPORT OF URIC ACID, PHOSPHORUS, AND ELECTROLYTES IN PATIENTS WITH NORMAL RENAL AND ADRENAL FUNCTION

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CHANGES in the urinary excretion of a variety of substances occur following the administration of ACTH or cortisone. It has been suggested that such changes in excretion may be due in part to alteration of renal function.[1-3] The present study was designed to test this hypothesis.

ACTH and cortisone have been administered to patients with normal renal and adrenal function. The hormones increased clearances of inulin and reduced the ratio of creatinine clearance to inulin clearance.[4] The present study demonstrates that ACTH and cortisone may induce changes in the excretion of uric acid, phosphorus, and electrolytes despite contrary changes in their serum concentrations or rates of filtration, or both. These data indicate that the metabolic changes that have been observed consequent to the administration of ACTH or cortisone may be due in part, at least, to alteration of renal tubular function.

MATERIALS AND METHODS

The clearance techniques used and the analytical methods employed for inulin and p-aminohippurate (PAH) are presented elsewhere.[4] The renal function of all patients was normal.[4] All the subjects were kept on a constant diet and were studied under basal conditions, in the postabsorptive state, at the same time of day. Observations during the periods of administration of hormone were made on the last day of each treatment period. The ACTH and cortisone were given intramuscularly every six hours, and the renal flow studies were begun within two hours after the last injection. Patient E. A., a 46-year-old white man with chronic alcoholism, received two doses of 25 mg. of ACTH,† followed by five doses of 50 mg. Patient D. S., a 35-year-old Negro man convalescing from acute rheumatic fever, received 25 mg. of ACTH per dose the first day, 70 mg. per dose the second day, and 105 mg. every six hours on the third day. Patient P. H., a 25-year-old white woman with facial der-

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Part of this work was done under contract with the Army Medical Research and Development Board, and was aided by a grant from the United States Public Service.

Presented, in part, before the American Society for Clinical Investigation, May 1, 1950 (Abs. *J. Clin. Investigation* **29**: 824, 1950), and at the Second Clinical ACTH Conference, Chicago, Dec. 9, 1950.

Received for publication, May 21, 1951.

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†ACTH supplied through the courtesy of Dr. John R. Mote of Armour Laboratories. Doses are relative to the standard LA-1A.

matitis, was given cortisone divided into aliquots that were given every six hours. She received a total of 75, 100, 125, 200, 275, 300, 325 mg. on seven successive days, and on the eighth day received 100 mg. two hours before the studies were begun. After a six-day interval this patient received 200 and 500 mg. per day on two successive days and was studied on the third day two hours after a single injection of 300 mg. of cortisone. Patient F. W., a 31-year-old man with Buerger's disease, involving only the extremities, received 200 mg. of cortisone per day in divided doses every six hours for eight days. After a ten-day interval he was given 750 and 700 mg. per day on two successive days, followed by a single injection of 150 mg. on the third day, before the study was begun.

Uric acid in the urine was determined by the method of Benedict and Franke,[5] and in the serum by the method of Folin.[6] Phosphorus in serum and urine was determined by the method of Fiske and Subbarow.[7] Determinations of the concentrations of sodium and potassium were made on the Perkin-Elmer flame photometer, using lithium as an internal standard.

The rate of filtration of any substance, or the "filtered load," was calculated as the product of its concentration in plasma and the inulin clearance. The percentage excretion of a filtered material (E/F) was considered to be equivalent to the ratio of its clearance to the simultaneous clearance of inulin.

Considerable variations in urine flow occurred during periods prior to the determination of Tm_{PAH} in both experiments performed in patient P. H., while cortisone was being given. It is probable that these fluctuations were due both to a marked subjective reaction to the procedure which could have affected both urine flow and clearance ratios, and to collection errors introduced as a result of these variations. The four pre-Tm_{PAH} clearance periods in each experiment were, therefore, calculated cumulatively as one long period.

All data were analyzed for statistical significance by the "t" test.[8] In all instances in which a function is said to have changed, the change was statistically significant and the "p" values less than 0.02.

RESULTS

The rates of excretion of uric acid, phosphorus, sodium, and potassium were determined before and during the administration of cortisone or ACTH. These rates were determined during the initial inulin and PAH clearance periods and also during the administration of the large amounts of sodium para-aminohippurate that are required for the determination of Tm_{PAH}. The data are presented in Tables I and II.

Rates of Excretion During Inulin and PAH Clearance Periods.—

Uric Acid: In three patients, E. A., D. S. and P. H., administration of ACTH or cortisone was associated with a decrease in the serum concentration of uric acid. In each, hemodilution, as estimated from the decrease in hematocrit, occurred during cortisone or ACTH administration, but was insufficient to account for the decrease in serum concentration. In patients E. A. and D. S., the clearance of inulin increased, but the filtered load of uric acid was diminished because serum concentration decreased. Nevertheless, minute excretion of urate increased to almost twice the control level.

During the administration of 1.0 Gm. of cortisone in two days to patient P. H., the rate of urate filtration decreased as a result of slightly diminished clearance of inulin and decreased serum concentration. However, the minute excretion was unchanged; hence the percentage excretion of filtered uric acid was greater (by 80 per cent) than it had been during the control test. Pronounced changes were not observed in F. W., in whom the total daily urinary excretion of uric acid was unchanged by the administration of cortisone.

Phosphorus.—Excretion of phosphate increased in all four patients, accompanied in three instances by decreased serum concentrations. In three patients, E. A., D. S., and P. H., the increments in minute excretion were approximately 65 per cent, 260 per cent, and 100 per cent, respectively, despite diminished filtered loads. In the fourth patient, F. W., the filtered load of phosphate did not change significantly, but minute excretion rose by more than 100 per cent.

Sodium.—With the exception of patient D. S., minute excretion of sodium during the administration of hormone diminished prior to determination of Tm_{PAH}. These decreased rates of excretion of sodium were accompanied by slight decreases in E/F. In two patients, E. A. and F. W., the combination of decreased sodium excretion and decreased E/F was accompanied by increased filtered load.

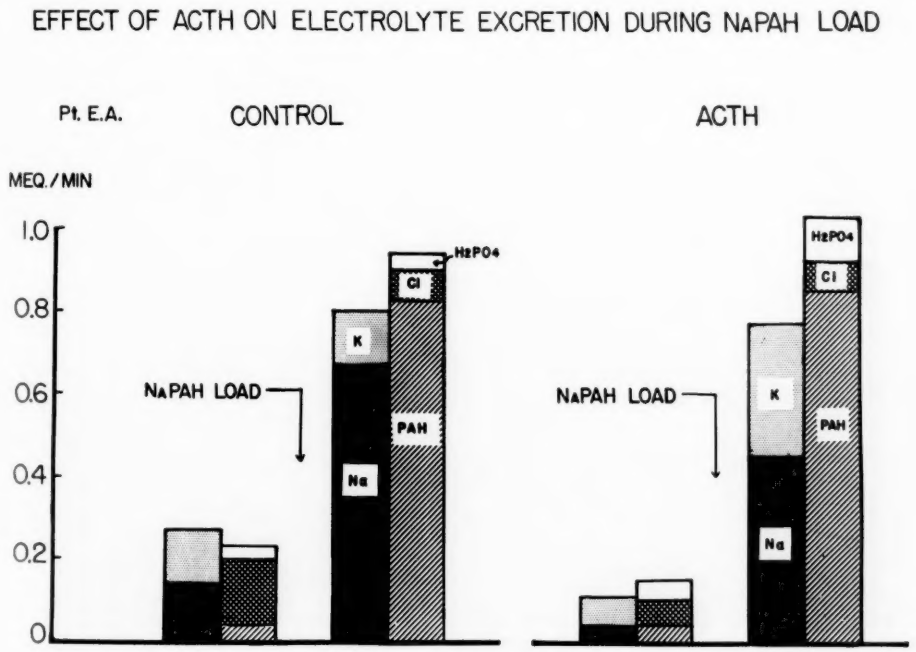

Fig. 1.

In D. S., sodium excretion, measured before determination of Tm_{PAH}, increased but this was associated with antidiuresis resulting from the intravenous infusion of 75 mg. of ACTH which was later found to contain antidiuretic hormone of the posterior pituitary.*

Potassium.—In three patients the rate of potassium excretion was not affected significantly by the hormones, although potassium filtration increased. In one patient, P. H., excretion increased 50 per cent, and the ratio of excreted to filtered potassium increased by 67 per cent during the administration of 1.0 Gm. of cortisone in two days.

*Armour Lot #8412. Pressor assay 0.04 unit per mg. ACTH.

TABLE I. EFFECT OF ACTH AND CORTISONE ON RENAL EXCRETION OF PHOSPHORUS AND URIC ACID IN PATIENTS WITH NORMAL RENAL AND ADRENAL FUNCTION†

	SUBJECT	TEST	SERUM CONC.‡ MG./100 ML.		EXCRETION MG./MIN.		CLEARANCE ML./MIN.		INULIN CLEARANCE ML./MIN.		FILTERED LOAD MG./MIN.		CLEARANCE/C_{IN} (E/F) %	
			BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD
URIC ACID	*E. A.*													
	S. A. = 1.65 M^2	1. Control	5.8	---	0.46 ± 0.03	---	9 ± 0.3	---	97	---	5.1 ± 0.9	---	9 ± 0.4	---
	Hct^1 = 48.6	2. A.C.T.H.§	1.8	---	0.71 ± 0.09	---	39 ± 2	---	134	---	2.4 ± 0.1	---	29 ± 2	---
	Hct^2 = 40.6													
	D. S.													
	S. A. = 2.23 M^2	1. Control	6.6	---	0.48 ± 0.04	---	7 ± 0.4	---	152	---	10.0 ± 0.7	---	5 ± 0.1	---
	$Hct.^1$ = 49.0	2. A.C.T.H.¶	3.7	---	0.82 ± 0.06	---	22 ± 2.5	---	203	---	7.9 ± 0.5	---	10 ± 0.2	---
	Hct^2 = 40.0													
	P. H.													
	S. A. = 1.69 M^2	1. Control	3.9	---	0.47 ± 0.05	---	12 ± 1.3	---	128	---	5.0 ± 0.1	---	10 ± 1.1	---
	Hct^1 = 33.3	2. Cortisone¶	3.1	---	0.37*	---	12*	---	85*	---	2.5*	---	11 ± 0.4	---
	Hct^2 = 31.7	3. Cortisone**	2.3	---	0.48*	---	21*	---	113*	---	2.8*	---	18 ± 0.3	---
	Hct^3 = 33.8													
	F. W.													
	S. A. = 1.82 M^2	1. Control	5.6	---	0.40 ± 0.05	---	7 ± 0.8	---	131	---	7.1 ± 0.4	---	6 ± 0.6	---
	Hct^1 = 46.6	2. Cortisone‡‡	4.7	---	0.36 ± 0.01	---	8 ± 0.3	---	157	---	7.4 ± 0.3	---	5 ± 0.3	---
	Hct^2 = 38.7													
PHOSPHORUS	E. A.	1. Control	2.9	2.8	0.78 ± 0.06	1.11 ± 0.10	27 ± 2.6	39 ± 7.6	97	83	2.8 ± 0.1	2.3 ± 0	28 ± 2.1	48 ± 4.4
		2. A.C.T.H.	1.6	1.6	1.29 ± 0.07	3.00 ± 0	79 ± 10	187 ± 18	134	110	2.2 ± 0.1	1.8 ± 0	60 ± 8.5	171 ±17
	D. S.	1. Control	4.8	5.0	0.52 ± 0.04	0.97 ± 0.11	11 ± 0.5	19 ± 2.0	152	116	7.5 ± 0.4	5.8 ± 0	7 ± 0.5	16 ± 1.4
		2. A.C.T.H.	3.3	3.5	1.86 ± 0.12	2.67 ± 0.13	58 ± 13	77 ± 2.6	203	191	6.5 ± 0.4	6.6 ± 0.2	28 ± 1	40 ± 0.1
	P. H.	1. Control	3.9	4.4	0.40 ± 0.06	1.34 ± 0.10	10 ± 0.7	30 ± 1.7	128	129	5.0 ± 0.2	5.8 ± 0.6	8 ± 0.3	24 ± 0
		2. Cortisone	4.0	3.8	0.68*	1.47 ± 0.02	17*	39 ± 1.6	85*	119	3.4*	4.5 ± 0.2	21 ± 0.2	33 ± 0.8
		3. Cortisone	3.7	3.3	0.78*	1.75 ± 0.12	21*	53 ± 1.2	113*	134	4.2*	4.3 ± 0.1	19 ± 0	39 ± 0.4
	F. W.	1. Control	4.4	4.4	0.42 ± 0.02	1.3 ± 0.2	10 ± 0.4	30 ± 1.8	131	110	5.8 ± 0.5	4.8 ± 0	7 ± 0.2	27 ± 1.1
		2. Cortisone	3.7	3.6	0.92 ± 0.02	1.6 ± 0	25 ± 1.3	44 ± 2.8	157	144	5.7 ± 0.2	5.2 ± 0.1	16 ± 0.8	32 ± 0.4

*Collection error suspected (see text).

†Values represent the mean of two to fourteen consecutive clearance periods, and, with the exception of the clearance of inulin and periods during which collection error is suspected, are given with the standard error of the mean.

‡Mean excretion/mean clearance.

§After 575 mg. ACTH given during three and one-half days.

‖After 980 mg. ACTH given during three and one-half days.

¶After 1.5 Gm. cortisone given during eight days.

**After 1.0 Gm. cortisone given during two days.

‡‡After 1.6 Gm. cortisone given during two days.

¶After 1.5 Gm. cortisone given during eight days.
**After 1.0 Gm. cortisone given during two days.
††After 1.6 Gm. cortisone given during two days.

TABLE II. EFFECT OF ACTH AND CORTISONE ON RENAL EXCRETION OF ELECTROCYTES IN PATIENTS WITH NORMAL RENAL AND ADRENAL FUNCTION†

	SUBJECT	TEST	SERUM CONC.‡ MEQ./L.		EXCRETION MEQ./MIN.		CLEARANCE ML./MIN.		INULIN CLEARANCE ML./MIN.		FILTERED LOAD MEQ./MIN.		CLEARANCE/C_{IN} (E/F) %		EXCRETION PAH^- MEQ./MIN.	
			BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD	BEFORE NaPAH LOAD	DURING NaPAH LOAD
SODIUM	E. A.	1. Control	139	140	0.15 ± 0.01	0.68 ± 0	1.1 ± 0.1	4.9 ± 0	97	83	13.3 ± 1.1	11.6 ± 0	1.1 ± 0.04	5.9 ± 0	0.04	0.80
		2. A.C.T.H.	129	132	0.08 ± 0.02	0.45 ± 0	0.6 ± 0.1	3.4 ± 0	134	110	17.7 ± 0.5	14.4 ± 0	0.4 ± 0.08	3.1 ± 0	0.04	0.80
	D. S.	1. Control	131	138	0.02 ± 0	0.71 ± 0.07	0.2 ± 0.1	5.1 ± 0.16	152	116	20.2 ± 0.4	16.3 ± 0.1	0.1 ± 0	4.3 ± 0.2	0.04	0.93
		2. A.C.T.H.	127	126	0.12 ± 0.02	0.53 ± 0.01	0.90 ± 0.16	4.2 ± 0.04	203	191	25.8 ± 1.4	24.2 ± 0.8	0.5 ± 0.08	2.2 ± 0	0.09	0.96
	P. H.	1. Control	144	147	0.23 ± 0.01	1.10 ± 0.06	1.6 ± 0.06	7.5 ± 0.8	128	129	17.9 ± 0.6	19.0 ± 1.4	1.2 ± 0.1	5.8 ± 0.2	0.06	1.03
		2. Cortisone	147	148	0.14*	0.65 ± 0.04	1.0*	4.4 ± 0.2	85	119	12.5*	17.5 ± 0.2	1.1 ± 0*	3.9 ± 0.1	0.04*	0.64
		3. Cortisone	143	143	0.12*	0.69 ± 0.04	0.8*	4.8 ± 0.1	113	134	16.2*	19.2 ± 0.7	0.8 ± 0*	3.6 ± 0.1	0.06*	0.87
	F. W.	1. Control	141	142	0.05 ± 0	0.69 ± 0.03	0.4 ± 0.04	4.9 ± 0.1	131	110	18.4 ± 0.8	15.7 ± 0.2	0.3 ± 0.02	4.4 ± 0.2	0.05	0.80
		2. Cortisone	145	143	0.02 ± 0	0.70 ± 0.02	0.2 ± 0.01	4.9 ± 0.1	157	144	22.7 ± 1.0	20.7 ± 0.2	0.1 ± 0.2	3.4 ± 0.1	0.04	1.06
POTASSIUM	E. A.	1. Control	5.3	3.9	0.10 ± 0.01	0.12 ± 0.01	19 ± 2.1	32 ± 0	97	83	0.5 ± .02	0.3 ± 0	20 ± 2	39 ± 2		
		2. A.C.T.H.	4.0	3.5	0.09 ± 0.01	0.30 ± 0.02	23 ± 0.6	86 ± 3.2	134	110	0.6 ± .02	0.4 ± 0.01	17 ± 1	78 ± 3		
	D. S.	1. Control	4.5	4.5	0.17 ± 0.02	0.18 ± 0	38 ± 2.5	40 ± 0	152	116	0.7 ± 0.04	0.5 ± 0	25 ± 1	34 ± 1		
		2. A.C.T.H.	4.1	3.7	0.13 ± 0.01	0.33 ± 0.01	32 ± 1.5	90 ± 2	203	191	0.8 ± 0.04	0.7 ± 0	16 ± 1	47 ± 1		
	P. H.	1. Control	4.4	4.2	0.08 ± 0	0.11 ± 0.02	18 ± 0.7	26 ± 1.2	128	129	0.5 ± 0.03	0.6 ± 0.05	15 ± 0	20 ± 1		
		2. Cortisone	4.4	4.2	0.07*	0.15 ± 0	17*	36 ± 0.7	85*	119	0.4*	0.5 ± 0.02	19 ± 0	31 ± 0		
		3. Cortisone	4.1	3.7	0.12*	0.20 ± 0.01	29*	54 ± 3.2	113*	134	0.5*	0.5 ± 0.02	25 ± 0	41 ± 0		
	F. W.	1. Control	4.3	4.1	0.12 ± 0.01	0.11 ± 0.01	28 ± 4	27 ± 1	131	110	0.6 ± 0.02	0.5 ± 0.01	22 ± 2	25 ± 1		
		2. Cortisone	4.6	4.1	0.11 ± 0	0.25 ± 0	24 ± 1	62 ± 1	157	144	0.7 ± 0.02	0.6 ± 0.02	16 ± 1	42 ± 1		

*Collection error suspected (see text).

†Values represent the mean of two to fourteen consecutive clearance periods, and, with the exception of the clearance of inulin, the excretion of PAH, and periods during which collection error is suspected, are given with the standard error of the mean.

‡Mean excretion/mean clearance.

Rates of Excretion During the Administration of NaPAH Loads.—(Fig. 1.) In all four patients loading with 10 to 15 Gm. of NaPAH prior to the administration of ACTH or cortisone markedly increased the excretion of sodium and the ratio of excreted to filtered sodium. Loading with NaPAH produced smaller increases in sodium excretion when patients E. A., D. S., and P. H. were receiving hormone than when they were not. In patient F. W., the magnitude of the increase in sodium excretion which resulted from loading with NaPAH was not significantly altered by the administration of cortisone. However, the hormone increased the excretion of PAH^-, so that proportionately less PAH^- was excreted with sodium than during the control test.

The changes in the excretion of sodium consequent to loading with NaPAH that occurred during the administration of hormone could not be explained by changes in the excretion of PAH, and were invariably accompanied by increased excretion of potassium. Both before and during the administration of ACTH or cortisone, loading with NaPAH usually resulted in slight decreases in the serum concentration of potassium, and in diminished or unchanged rates of filtration of this cation. Before the hormones were given, little or no change in potassium excretion occurred while Tm_{PAH} was being determined. However, in all cases during the administration of ACTH or cortisone, loading with NaPAH markedly augmented potassium excretion, and E/F increased by 64 to 360 per cent.

In all four patients, increased excretion of phosphate occurred during determination of Tm_{PAH}, both prior to and during the administration of ACTH or cortisone.[9] The increments which occurred consequent to infusion of NaPAH during the administration of ACTH were much greater than those which occurred during control tests. In the two patients who received cortisone, the increases in phosphate excretion which resulted from loading with NaPAH were not significantly altered by treatment with hormone.

While patient E. A. was receiving ACTH, during loading with NaPAH, clearances of phosphate were 190 per cent and 150 per cent of the simultaneous clearance of inulin in two consecutive clearance periods, suggesting that secretion of phosphate was occuring in this patient.

DISCUSSION

In confirmation of earlier reports[1, 10-12] it was found that in three of four patients the administration of ACTH or cortisone resulted in marked increases in the excretion of uric acid, despite lower serum concentrations. Moreover, simultaneous determination of inulin clearance indicated that the rate of glomerular filtration of uric acid was also reduced. The ratio clearance of urate to clearance of inulin was increased, confirming earlier data using creatinine clearance as a measure of glomerular filtration.[13]

If uric acid is totally filterable at the glomerulus[14-16] and is reabsorbed by the tubules,[16, 17] these data suggest that the uricosuric effect of ACTH and cortisone is the result of altered renal tubular transport. Further evidence for a primarily renal mechanism for the uricosuric effect of ACTH and cortisone is furnished by the observation that the administration of adrenal cortical extract

to nephrectomized animals does not alter the rate of accumulation of uric acid in the serum.[18] It is of interest in this connection that the uricosuric effect of ACTH may be partially or totally abolished by the prior administration of carinamide which is itself a uricosuric agent that affects tubular transport mechanisms for uric acid.[19]

The marked increases in the excretion of phosphorus which occurred in these patients despite diminished or constant rates of filtration of phosphorus, and despite decreases in serum inorganic phosphate concentrations, suggest that ACTH and cortisone reduce tubular reabsorption or increase tubular secretion of phosphate, or both. In this respect, the action of the adrenal steroids is similar to that of parathormone.[20] The administration of ACTH does not induce hypocalcemia, however, and hypophosphatemia rather than hyperphosphatemia occurred in these patients. Thus, the two conditions considered to result in parathyroid activation cannot be invoked to explain the increased phosphate excretion in these patients.[20, 21] There is no evidence that the adrenal steroids produce parathyroid activation via other mechanisms. It appears likely, therefore, that ACTH and cortisone may decrease tubular reabsorption of phosphorus as does parathormone. Such changes in phosphate reabsorption may account for losses of phosphorus exceeding those of nitrogen, as well as for the appearance of negative phosphorus balance in the absence of negative nitrogen and calcium balances.[10, 22]

There is ample evidence that the adrenal cortical hormones increase the net tubular reabsorption of sodium.[23-25] The data herein presented are in agreement with this concept, and have received recent confirmation.[26]

Similarly, a direct effect of the adrenal steroids on tubular function could explain the occurrence of negative balances of potassium exceeding those expected from the magnitude or direction of the nitrogen balance.[10, 22]

The administration of hypertonic sodium solutions may result in increased excretion of potassium in normal subjects.[26] In the patients presented herein, however, little or no change in potassium excretion followed the administration of large loads of NaPAH prior to the administration of ACTH or cortisone, but markedly increased excretion of potassium occurred during the determination of Tm_{PAH} while the patients were receiving hormone. It is noteworthy that in three patients the tendency to excrete more potassium during the administration of hormone was not apparent until after the NaPAH load was given. During the administration of ACTH of cortisone potassium excretion reached 24 to 38 per cent of the excretion of PAH during loading periods.

During the administration of NaPAH, the excretion of large quantities of the nonreabsorbed anion of this salt may have brought about the excretion of large quantities of cation. Inasmuch as ACTH and cortisone decrease sodium excretion, it is possible that the additional excretion of anion during loading with NaPAH necessitated a still greater increase in the excretion of potassium. Under such circumstances, the effects of adrenal steroids on the excretion of potassium would be secondary to the effects of the hormones on excretion of sodium. Possibly related to these findings is the observation that in patients with the

nephrotic syndrome, in whom reabsorption of sodium is increased, the administration of large quantities of sodium salts of rapidly excreted anions results in the excretion of large amounts of potassium.[27, 28] Furthermore, it has recently been reported that DOCA does not increase potassium excretion when given to patients receiving a low sodium diet in whom maximal sodium excretion is already occurring.[29] The possibility that the effect of ACTH or cortisone on the excretion of potassium is secondary to the effect of the hormones on the excretion of sodium is consistent with the recent suggestion that increased potassium excretion may, under certain circumstances, involve intratubular exchange with sodium.[30]

SUMMARY AND CONCLUSIONS

In patients with normal renal and adrenal function, the administration of ACTH or cortisone has resulted in changes in the rates of excretion of uric acid, phosphorus, sodium and potassium which appeared to be independent of changes in the serum concentrations and rates of filtration of these substances.

This suggests that ACTH and cortisone may alter the renal tubular transport of these substances. Such changes in renal function, induced by ACTH or cortisone, may be in part responsible for the metabolic effects of these hormones.

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COMPARATIVE EFFECTS OF HEPARIN AND PARITOL-C UPON BLOOD COAGULATION AND PROTHROMBIN TIME AND UPON THE PLASMA ANTITHROMBIN LEVEL

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THE demonstrated value of anticoagulant therapy and the development of new anticoagulant agents require a careful review of the methods for measuring alteration of the clotting mechanism. We have studied the effects of heparin and Paritol-C† on the plasma antithrombin level, prothrombin time, and coagulation time and wish to present evidence that the determination of the plasma antithrombin level offers a dependable and practical means of evaluating the effects of these drugs.

Studies on the anticoagulant properties of Paritol have been reported recently by Seifter and Begany,[1] Sorenson, Seifter, and Wright,[2] and Sorenson and Wright.[3] These studies indicate that Paritol may be administered safely and that it compares favorably with heparin as a rapidly acting anticoagulant agent. In certain respects regarding uniformity of action and period of activity it would appear to be even preferble to heparin.

Both drugs influence the blood coagulation time directly, but this effect has not proved generally satisfactory as a guide for their administration. Dissatisfaction has arisen mainly because of variations in the technical methods for determining the coagulation time, the subjectivity of these tests to circumstantial influence, and the time element involved. Both heparin and Paritol affect the prothrombin time, but this effect is not of sufficient degree to permit prothrombin determinations to be used as a dependable guide for their administration. In the effort to find a more trustworthy guide for the clinical application of these drugs we have studied their influence on the plasma antithrombin level in comparison with the coagulation time and prothrombin time.

METHODS

Coagulation Time, "CT" (Modification of the Lee-White Technique[4]).—

Using a dry syringe and 20-gauge needle, a clean venipuncture was made and the tourniquet promptly released. Not less than five milliliters of blood was drawn, and exactly one milliliter was placed in each of three dry test tubes (12 mm. by 75 mm.) and the procedure carried out at room temperature. Tilting of the first tube to the horizontal position at each half-minute interval was begun three minutes after blood first appeared in the syringe, and continued until the flow of the blood ceased. At this point tilting of the second tube was started

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This work was made possible through a grant from the M. D. Anderson Foundation, with additional support from private sources.

Received for publication April 12, 1951.

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†A polysulfuric acid ester of polyanhydromannuronic acid supplied by Wyeth Incorporated, Philadelphia, Pa.

and continued to a similar stage of clotting when tilting of the third tube was begun and carried out in the same manner. The time elapsing from the instant blood appeared in the syringe to that at which such clotting occurred in the third tube was designated as the CT. Normal values determined by this method varied widely between the extremes of thirteen and twenty-five minutes.

For the purpose of this study, the range of normal control in the CT was considered as lying within 15 to 22.5 minutes. In using this method to portray the anticoagulant action of heparin or Paritol, approximately two and one-half times the initial CT was considered as indicating a desirable therapeutic effect. Thus, the range of desired effect in the CT lay within thirty-five to fifty-five minutes.

Prothrombin Time, "PT" (Modification of Quick's one-stage method[5]).—

Normal control values on whole plasma, using rabbit lung thromboplastin (Maltine Co.), were determined to fall within a range of twelve to fifteen seconds.

In the routine application of this test as a guide for anticoagulant therapy a PT of twenty-two to thirty-eight seconds was considered as lying within the range of desired effect.

Plasma Antithrombin Test, "AT" (Modification of the Kay and Balla test[6]).—

The principle of this test is based on the knowledge that plasma normally contains a sufficient amount of antithrombic substance to inhibit a specific amount of thrombin and that clotting can occur only when the thrombin present exceeds this amount.

Preparation of the plasma: To a graduated centrifuge tube containing one milliliter of a tenth molar sodium oxalate solution nine milliliters of blood was added and mixed well. Centrifugation was carried out at 1,700 r.p.m. for ten minutes. The plasma was removed and stored at 5° C. until tested. Plasma so prepared was also used for the determination of the PT by the method referred to.

Preparation of the thrombin: Units of thrombin referred to in this study are subject to question by virtue of technical errors in dilution and inherent percentage inaccuracies in thrombin assaying. However, we have used the thrombin unit designation as a relative guide for dilution purposes, and have used the behavior of prepared plasma from normal adults as a reference point for evaluating all solutions of diluted thrombin. Each lot of thrombin was standardized against a number of plasma specimens from normal subjects.

A *stock solution* of thrombin was prepared by dissolving one ampule (labeled 1,000 units) of Thrombin Topical* in distilled water so that each milliliter contained approximately 100 units. This solution remained stable for a week and for convenience was stored in the frozen state in closed tubes containing one milliliter aliquots.

A *primary solution* of thrombin was made from the stock solution by diluting the latter with distilled water (1:10) to contain 10 units of thrombin per milliliter. The primary solution was diluted with distilled water (1:10) for the *test solution* which contained a thrombin concentration of one unit per milliliter. Progressive unit concentrations of thrombin to carry out the test were then made by diluting the test solution with distilled water so that each 0.1 ml. of solution contained 0.01, 0.015, 0.02, 0.03, 0.04, 0.05, and 0.08 unit thrombin, respectively. Solutions of higher concentrations of thrombin were obtained by adding appropriate amounts of distilled water to the primary solution (10 units per milliliter).

Test procedure: A 0.5 ml. aliquot of the plasma to be tested was placed in each of five (12 mm. by 75 mm.) pyrex tubes and to these tubes serially was added 0.1 ml. of each of five selected unit concentrations of thrombin. Incubation at 37° C. was carried out for thirty minutes and the presence or absence of clot formation was immediately determined by gentle agitation of each tube in a bright light. In ascending concentrations the end point was determined as the unit concentration of thrombin in the first tube containing a definite fibrin strand or clot. When an end point could not be determined in the series chosen, a higher or lower series of concentrations was selected for testing as indicated. Care was taken not to mistake flakes of protein, lint, or dust for fibrin strands.

*Supplied by Parke, Davis, & Company. Within 5 per cent margin of error the thrombin content of each ampule will be supplied by Mr. Eugene C. Loomis, Parke-Davis Research Laboratory, Detroit 32, Michigan, upon receipt of the lot number of the ampule.

According to this procedure, the plasma of normal adults showed an end point of antithrombic activity falling within a range of 0.02 to 0.05 unit thrombin concentration. As a guide for therapy two to three times the initial control AT titer was considered a desirable range of anticoagulant effect, which is comparable to the accepted therapeutic range as portrayed by the CT and the PT. Thus, a sufficient antithrombic effect was induced to inhibit at least 0.08 unit and not more than 0.5 unit thrombin as a dependable indication of anticoagulant control.

These relatively minute fractional unit concentrations of thrombin have been employed for the purpose of investigating the normal levels of plasma antithrombin and the effects of the antithrombin agents heparin and Paritol on these levels.*

It proved advisable to determine the AT level within four hours from the time the blood was drawn. Samples stored from approximately four to sixteen hours for testing tended to show relatively higher levels and occasionally an originally low titer was obscured. When samples were stored for more than sixteen hours, however, the AT level tended to fall again and in this instance an occasional false low initial titer was suggested. These minor variations in the AT level due to storage of the plasma did not appear to be of significance in the use of the test as a guide for therapy.

RESULTS

Plasma antithrombin determinations were made on one hundred healthy control subjects who presented no evidence of vascular disease and on fifty-five patients suffering from coronary artery disease and thromboembolic diseases of various types (Table I).

TABLE I. ANTITHROMBIN LEVELS ON 100 NORMAL CONTROL SUBJECTS AND 55 CASES OF THROMBOEMBOLIC DISEASE

UNIT THROMBIN	0.01	0.015	0.02	0.03	0.04	0.05	0.06
100 Normal controls	0	0	6	45	43	6	0
55 Cases thromboembolic disease	0	2	7	31	12	3	0

The plasma of normal subjects showed end points falling consistently within the range of 0.02 to 0.05 unit concentrations of thrombin. Eighty-eight per cent of these end points fell about equally at the 0.03 and 0.04 unit concentration levels. The results obtained in the fifty-five cases of thromboembolic disease portrayed a relative decrease in the antithrombin titer. A statistical analysis of the two groups indicates a difference in the end point means equal to 3.92 times the probable error which is significant for the group but could not be considered significant for the individual case. A critical clinical evaluation of these results will be reported in a subsequent paper.[7]

Determination of the antithrombin levels on the plasmas of both normal and ill subjects who had received either heparin or Paritol demonstrated a consistent rise in titer proportional to the amount of the drug administered. Detailed graphs of these effects, for single and repeated doses of both heparin and Paritol, together with the comparative effects on the CT and PT are presented in Figs. 1 and 2.

*For routine laboratory use the test may be greatly facilitated by reducing the number of concentration levels employed. This is accomplished by serially diluting the primary solution (10 units per milliliter) so that six tubes contain 0.50, 0.25, 0.125, 0.06, 0.03 and 0.015 unit, respectively.

By this method the end point of antithrombic effect in plasma from normal adults falls in the tubes containing 0.03 or 0.06 unit thrombin concentrations. Therefore, a desirable therapeutic anticoagulant effect would establish an end point in the tube containing 0.125 unit thrombin.

Case 1, Fig. 1, received 1 mg. per kilogram Liquaemin Heparin (Roche) and the CT, PT, and AT were determined at the ten and thirty minute, and at the two, four, six, eight, twelve, and twenty-four hour periods. A prompt and marked rise in the CT is observed at ten minutes, and is well maintained for at least another twenty minutes. However, at the two-hour period a sharp drop approaching the range of normal has occurred, and at four hours a complete lack of heparin effect is indicated. The response in the PT is similar to that in the CT, but much less pronounced and somewhat more prolonged. The AT level remains above the range of normal for a period of approximately twelve hours.

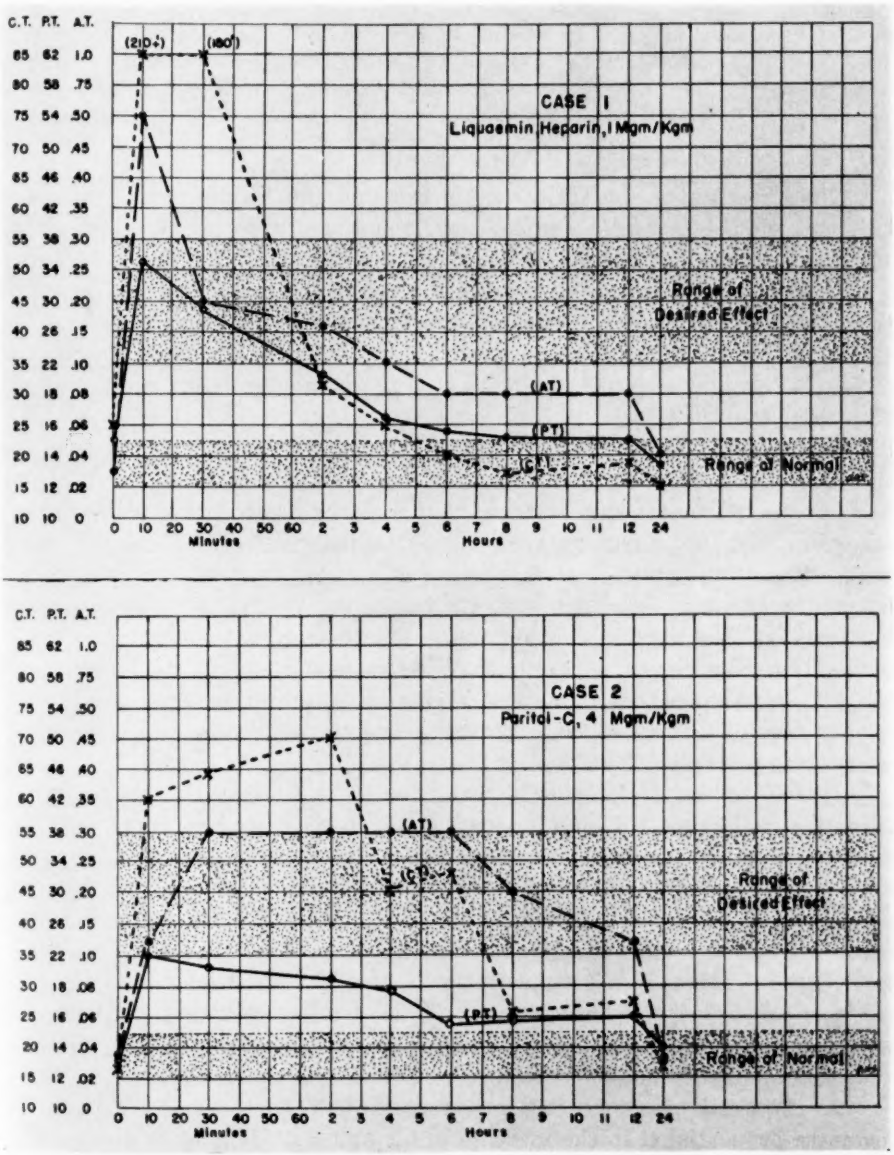

Fig. 1.—Comparison of the effects of separate intravenous injections of Liquaemin Heparin and Paritol-C on the coagulation time, prothrombin time and antithrombin level. (CT = coagulation time in minutes; PT = prothrombin time in seconds; AT = plasma antithrombin in units of thrombin.)

When 4 mg. per kilogram Paritol was administered intravenously (Case 2, Fig. 1), and similar periodic observations made the effect on the CT appears prompt but less exaggerated and more prolonged than that of heparin. With the amount of drug administered in this case the PT shows a slightly less abrupt response. The AT titer rises immediately into the range of desired effect, reaching a maximum within thirty minutes, and remains effective for a period of at least twelve hours.

Case 1, Fig. 2, received repeated subcutaneous injections of Depo-heparin (Upjohn) at twelve-hour intervals, and the effects under consideration determined just before each injection. It may be seen that the AT level is maintained within the range of desired effect by varying the dosage of the drug as indicated while the CT responds erratically. When the effect of Paritol was prolonged by repeated injections at twelve-hour intervals, Case 2, Fig. 2, the AT level dependably reflects the amount of the drug administered while the CT response is undependable. The PT in both cases, Fig. 2, consistently shows the effect of the drug administered, but in the case receiving Paritol evidence of a cumulative effect is suggested.

DISCUSSION

The exact nature of antithrombin remains undetermined and its specific influence on the balanced clotting mechanism continues to be obscure. The effects of such a factor have been repeatedly demonstrated, however, and recently Kay and associates[6] have devised a relatively simple test for measuring the "plasma antithrombin." In this study the terms in common use have been employed for the purpose of presenting a practical quantitative modification of Kay's test and a useful application of the principle in the control of anticoagulant therapy.

Heparin has been shown to affect the plasma antithrombin level, and therefore has been described as an antithrombic agent.[8] Klein and Seegers[9] have presented convincing evidence that heparin acts to prevent the union of thrombin with fibrinogen rather than as an inactivator of thrombin, per se. Regardless of the manner of the action of heparin in vivo, the in vitro anticoagulant effect is dependably measured by the plasma antithrombin determination as described. The action of Paritol can be demonstrated as dependably. Experience with the test, when performed in conjunction with the CT, has shown that it may be used as a safe and accurate guide to the proper administration of these short-acting anticoagulants, and consequently we no longer employ the CT.

Hurn, Barker, and Mann[10] have reported an increase in the serum antithrombin following the administration of Dicumarol, and Owen and Bollman[11] are in general agreement with his finding but state that Dicumarol does not measurably influence the plasma antithrombic action. We have been unable to demonstrate any measurable influence on the plasma antithrombin level with the use of either Dicumarol, Tromexan* or BL-5.† We therefore have utilized this test in conjunction with the determination of the prothrombin time to differentiate between the effects of the short-acting and long-acting anticoagulants when administered simultaneously. A description of this application of the test is to be presented in a subsequent paper.[7]

In the cases presented it is apparent that the increase in AT is not consistently proportional to the increase in CT and PT. It is to be observed that

*Supplied by Geigy Company.

†4-Hydroxycoumarin Anticoagulant No. 63; supplied by Abbott Laboratories.

the AT remains elevated or abnormally high for a longer period of time than the CT following the administration of either heparin or Paritol. This indicates that the anticoagulant effect of these drugs is maintained for a longer period of time than that assumed when their action is measured by the usual methods of determining the CT. This probability has been discussed by other investigators in regard to the action of heparin,[12, 13] and deTakats[14] has re-

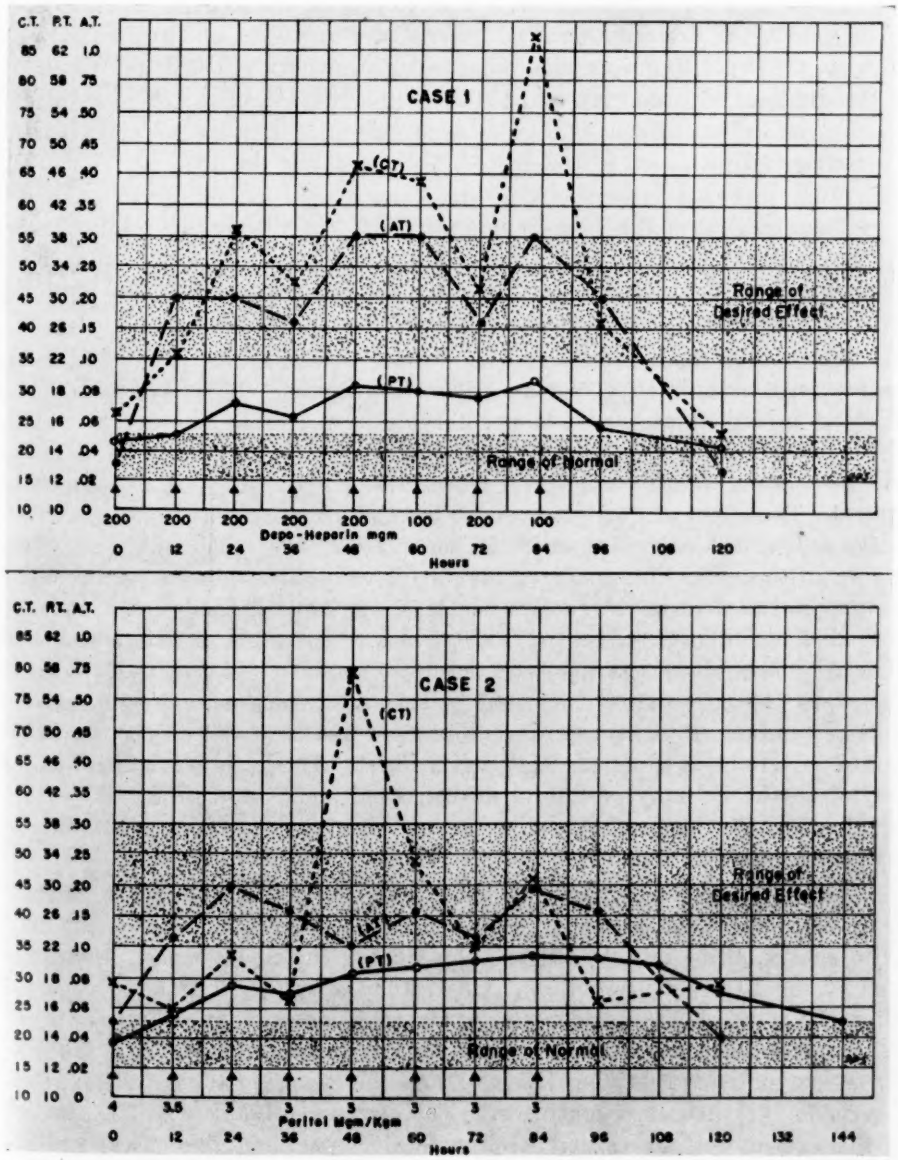

Fig. 2.—Comparison of the effects of repeated injections of Depo-heparin and Paritol on the coagulation time, prothrombin time and antithrombin level. (*CT* = coagulation time in minutes; PT = prothrombin time in seconds; AT = plasma antithrombin in units of thrombin.)

ported a method for determining a "sensitized clotting time" which offers a more critical measure of its anticoagulant effect. A direct comparison of the sensitized clotting time, using one gamma of powdered heparin as the sensitizing dose, with the plasma antithrombin level indicates that the latter is more sensitive and more consistent in its response to the influence of heparin.

Two hundred milligrams of Depo-heparin (Upjohn) or Heparin Repository,* administered subcutaneously, was found to produce an antithrombic effect within the desired therapeutic range for a period of twelve hours in most cases. Depending upon the individual's sensitivity to heparin the amount of the drug, or the time interval between injections, was varied to maintain the desired effect. Even when the dose and time factors were stabilized, there occurred instances in which the sensitiveness to the drug or the rate of absorption appeared to become altered. Thus, after a satisfactorily stabilized period, one subject might show a cumulative effect, while another might gradually lose anticoagulant control. It was found that pain or the development of a hematoma at the site of injection often limited the use of these forms of heparin to not more than two or three consecutive injections.

Four milligrams per kilogram of Paritol was found to produce a satisfactory antithrombic effect for a period of approximately twelve hours. On repeated injections at twelve-hour intervals it was often found that a dose of 3.5 mg. per kilogram, or even 3 mg. per kilogram, was adequate to maintain the plasma antithrombin level within the desired range of anticoagulant control. The effect of Paritol appeared to be much more consistently reflected in the antithrombin level than in the coagulation time. The maximum effect in the antithrombin titer occurred regularly at the thirty-minute period, while the maximum effect in the coagulation time was unpredictable. No tendency toward a cumulative effect of Paritol has been noted by Sorenson and Wright.[3] This study has not reflected any cumulative effect of the drug in regard to the coagulation time and plasma antithrombin but, in most subjects, a gradual rise in the prothrombin time was observed on repeated controlled injections as illustrated in Case 2, Fig. 2. The cumulative effect of the Paritol on the PT may be due to an increased blood level of Paritol with a direct effect on the prothrombin or to an interference of the production of prothrombin in the liver.

Our experience with Paritol emphasizes the recommendations for caution in the administration of the drug. Care has been exercised to give the drug quite slowly, three minutes being required for the injections of each milliliter of the 10 per cent solution. One rather severe local reaction occurred when the solution was accidentally extravasated outside the vein. Both immediate and delayed systemic reactions have been encountered. In a group of thirty-four patients receiving 125 injections the following toxic reactions were observed: (1) nausea, twenty cases; (2) dizziness, eight cases; (3) tingling of the extremities, six cases; (4) hypotensive reactions, five cases; and (5) sensitivity reactions, three cases. Most of these reactions could be considered

*Supplied by Lederle Laboratories.

as mild, but the hypotensive and sensitivity reactions assumed rather alarming proportions which could not be ascribed to over-rapid administration of the drug. On the basis of these observations it appears advisable to avoid the use of Paritol in subjects who evidence shocklike symptoms or in those who have manifested previous sensitivity reactions.*

SUMMARY

A practical method for quantitatively determining the plasma antithrombic activity has been described.

Experience with this test indicates it to be more sensitive and less erratic than determination of the coagulation time or the "sensitized clotting time."

The anticoagulant effects of heparin and Pariol are accurately reflected in the level of the plasma antithrombin as determined.

Neither Dicumarol, Tromexan, nor BL-5 produce a significant effect on the plasma antithrombin level.

It is suggested that the administration of Paritol be avoided in shock states and in known sensitivity reactors.

Appreciation is expressed for the advice and aid given in this work by Dr. Moise D. Levy, Sr., Chief of Medicine, and Dr. Joe R. Gandy, Chief Surgeon, and for the cooperation of the entire Hospital Personnel.

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*Experience with recent lots of an improved 5 per cent solution of Paritol-C has been much more satisfactory, and only minor reactions have been observed.

EXPERIENCES WITH HEPARIN: COMPARATIVE EFFECTS OF INTRAVENOUS AND SUBCUTANEOUS ADMINISTRATION IN THE SAME INDIVIDUALS

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IT IS known that variable responses of the coagulation time of whole blood occur in different individuals following intravenous injections of fixed doses of heparin.[1-4] Heparin tolerance also appears to vary from time to time in the same patient following major operations and during the active phases of certain thrombo-embolic diseases. DeTakats[2] has suggested that the patient's response to a heparin tolerance test might be used to predict the amount of heparin required for treatment.

There have been many recent reports regarding the subcutaneouos administration of heparin in slowly absorbed vehicles, such as one containing 18 per cent glycerine and 8 per cent glucose. There has been disagreement, however, as to the optimal dosage and frequency of administration of heparin in such media for purposes of producing an adequate but not excessive anticoagulant effect. Loewe[5] felt that 300 to 400 mg. of such a preparation might give a coagulation time three times normal for forty-eight hours or more. Bauer and his co-workers[6] experienced unfavorable results with this method and dosage. DeTakats[7] used 400 mg. doses in various combinations with and without vasoconstricting agents, and found that an effective prolongation of the coagulation time of capillary blood lasted for periods ranging from thirteen to forty-eight hours. Marple and Wright[8] have said that 200 mg. given twice daily may be used effectively during the first days of dicumarol therapy before the action of the dicumarol has become established. Goodwin and Macgregor,[9] in reporting on the use of heparin in Pitkin's menstruum administered subcutaneously to twenty-four patients, stated that wide variations in coagulation times occurred from patient to patient and in the same patient from day to day. They felt that the minimal effective dose was 200 mg. every twelve hours and that the average effective dose was 200 mg. alternating with 400 mg. every twelve hours. To our knowledge there has been no report of a study of the comparative effects of heparin given intravenously and subcutaneously to the same individuals.

We felt that it would be of interest to determine if the ten-minute response to heparin given intravenously could be used to predict the optimal dose, degree of effect and duration of effect of heparin given subcutaneously in a slowly absorbed medium.†

Received for publication May 1, 1951.

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†Depo-heparin supplied by The Upjohn Company.

METHODS OF STUDY

The comparative effects of single doses of heparin administered intravenously and subcutaneously were studied in twenty subjects, with their permission. One of these was a paraplegic without evidence of peripheral vascular disease (T); three were healthy adults (B, C, and D); four had experienced recent acute myocardial infarctions (A, E, G, and K); and twelve had evidence of various types of peripheral vascular disease complicated by thrombosis (F, H, I, J, L, M, N, O, P, Q, R, and S). Single twenty-four-hour studies were done in fourteen of the twenty subjects. Two studies were done in five subjects, and four studies were done in one subject. Vasoconstricting agents were not used with the subcutaneously administered heparin. Each 200 mg. of subcutaneously administered heparin was injected into a separate site in the upper part of the arm or in the buttock.

Coagulation times of whole venous blood were recorded before and ten minutes after heparin had been given intravenously and at four, twelve, eighteen, and twenty-four hours or more after its subcutaneous administra-

TABLE I. TWENTY-FOUR-HOUR STUDIES ON SUBJECTS GIVEN HEPARIN SUBCUTANEOUSLY

SUBJECT	COAGULATION TIME IN MINUTES (23°-26° C.)		DOSE OF HEPARIN SUBCUTANEOUSLY IN MG.	COAGULATION TIME IN MINUTES 4 HOURS LATER	DURATION OF EFFECT ON COAGULATION TIME IN HOURS	
	CONTROL	10 MINUTES AFTER 50 MG. OF HEPARIN INTRAVENOUSLY			LAST TEST WHICH GAVE RESULT TWICE CONTROL OR GREATER	FIRST TEST WHICH SHOWED RETURN TO CONTROL TIME
G	6	11	400	13	4	19
	*	*	600	17	11	24
	7	14	600	21	11	24
	*	*	400	32	11	24
P	6	14	600	32	18	Above control at 24
R	4	15	600	20	18	24
T	6	16	600	47	24	36
E	6	19	400	39	12	24
	*	*	600	60+	18	24
O	7	20	600	45	12	24
B	7	21	200	28	4	11
	7	45	400	16	14	24
N	5	22	600	17	12	24
F	5	23	400	31	18	24
	*	*	600	44	18	24
Q	7	23	600	60+	18	36
H	5	25	400	60+	18	24
	*	*	600	60+	18	Above control at 24
K	6	26	400	35	24	Above control at 24
	*	*	600	60+	24	Above control at 24
J	7	26	400	30+	12	24
I	7	27	400	29	24	36
S	6	28	600	60	15	24
L	10	29	400	45+	4	24
M	7	31	600	20	12	24
A	5	35	400	20	24	Above control at 24

*Not repeated between two consecutive studies.

tion. In a few instances, as noted in Table I, the tests were done at eleven, fourteen, and fifteen hours after injection instead of twelve hours thereafter, and nineteen hours after injection instead of eighteen. Two determinations of coagulation time were made on each sample of blood tested. One portion was tested in the Barker coagulochronometer[10] in which the environmental temperature was 37° C. and the rate of tipping the tube was kept constant. Another portion was tested at room temperature (23° to 26° C.) by the method of Lee and White.[11]

There was a fairly high positive correlation of the results of the tests done by the two methods when the coagulation times were prolonged by heparin. Usually the Lee-White determination gave a longer time, as would be expected from a test done in a lower environmental temperature, but this was not invariably true. For purposes of illustration, only the Lee-White determinations are given in the tables, since this is the method most commonly used for doing the test.

All technical work was done by one of us (Hauch), and attempts were made to control and standardize, as carefully as possible, the many factors which are known to influence the results obtained when the coagulation time of whole blood is determined. In spite of all possible precautions, there were some inconsistencies.

For purposes of analysis, an effective coagulation time was arbitrarily assumed to be at least twice that of the control test. To our knowledge the minimal degree of prolongation of the coagulation time by heparin which indicates an anticoagulant effect sufficient to prevent thrombosis has not been established. This minimal effect probably varies for different individuals and diseases. Since any slight increase of the coagulation time above the control coagulation time may be significant, the time elapsing before the result of the test had returned to the control level was also recorded.

OBSERVATIONS

The coagulation times ten minutes after the intravenous injection of 50 mg. of heparin varied from eleven to forty-five minutes.

In three normal subjects tested early in the study the duration of twice normal coagulation times following subcutaneous injection of 200 mg. of heparin was less than twelve hours, and the coagulation time had returned to the control level in eighteen hours. For this reason it was considered that larger doses would be necessary to produce effects lasting twenty-four hours, particularly in subjects with thrombosis.

A summary of results in eleven studies on ten subjects when 400 mg. of heparin was given subcutaneously and in fourteen studies on thirteen subjects when 600 mg. of heparin was given subcutaneously is presented in Table I. In both series the variability of response among the different subjects to both heparin given intravenously and heparin given subcutaneously is evident. There was no positive correlation between the degree of response ten minutes after heparin was given intravenously and the degree of response four

Coagulation time in minutes

hours after it had been administered subcutaneously. Also, there was no correlation between either of these and the control coagulation times. The correlation between the degree of response to heparin given intravenously and duration of effect of heparin given subcutaneously was low. On the basis of these data it appears that the response to heparin administered intravenously cannot be used clinically to predict either the degree or duration of response to heparin given subcutaneously in a slowly absorbed medium.

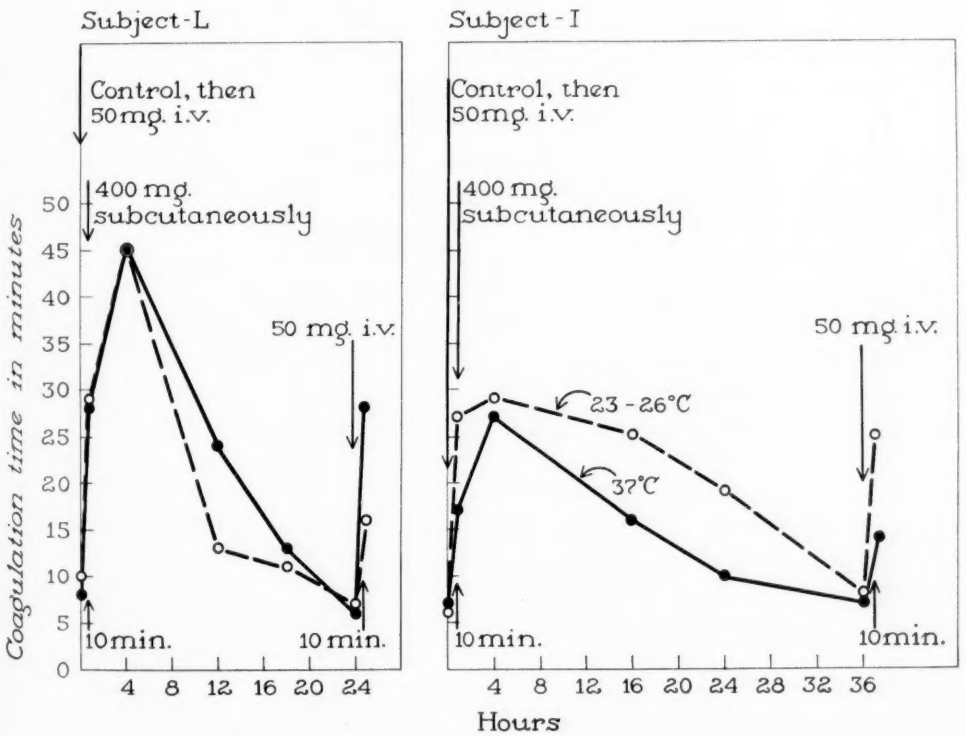

Fig. 1.—Responses of coagulation time to heparin administered intravenously and subcutaneously to a man with ileofemoral thrombophlebitis and pulmonary embolism (subject L) and a woman with thrombophlebitis in a superficial varix (subject I). In both subjects the response to heparin administered intravenously was essentially unchanged before and after the subcutaneous administration of heparin. The duration of effect of heparin given subcutaneously was much shorter in the subject with the more extensive thrombosis.

A summary of effects of different doses of heparin administered subcutaneously to six different subjects (G, E, B, F, H, and K) is given in Table I. It will be noted that after the larger dose the intensity of effect was greater than after the smaller dose in three of these subjects (E, F, and K), and the duration of effect was prolonged slightly in three others (G, B, and H).

The prolongation of coagulation time to twice normal or greater continued for eleven hours or more in all but two of the twenty-five studies on subjects receiving 400 or 600 mg. of heparin subcutaneously. In only thirteen of the twenty-five did this degree of effect continue for eighteen hours or longer, and in only five did it last twenty-four hours.

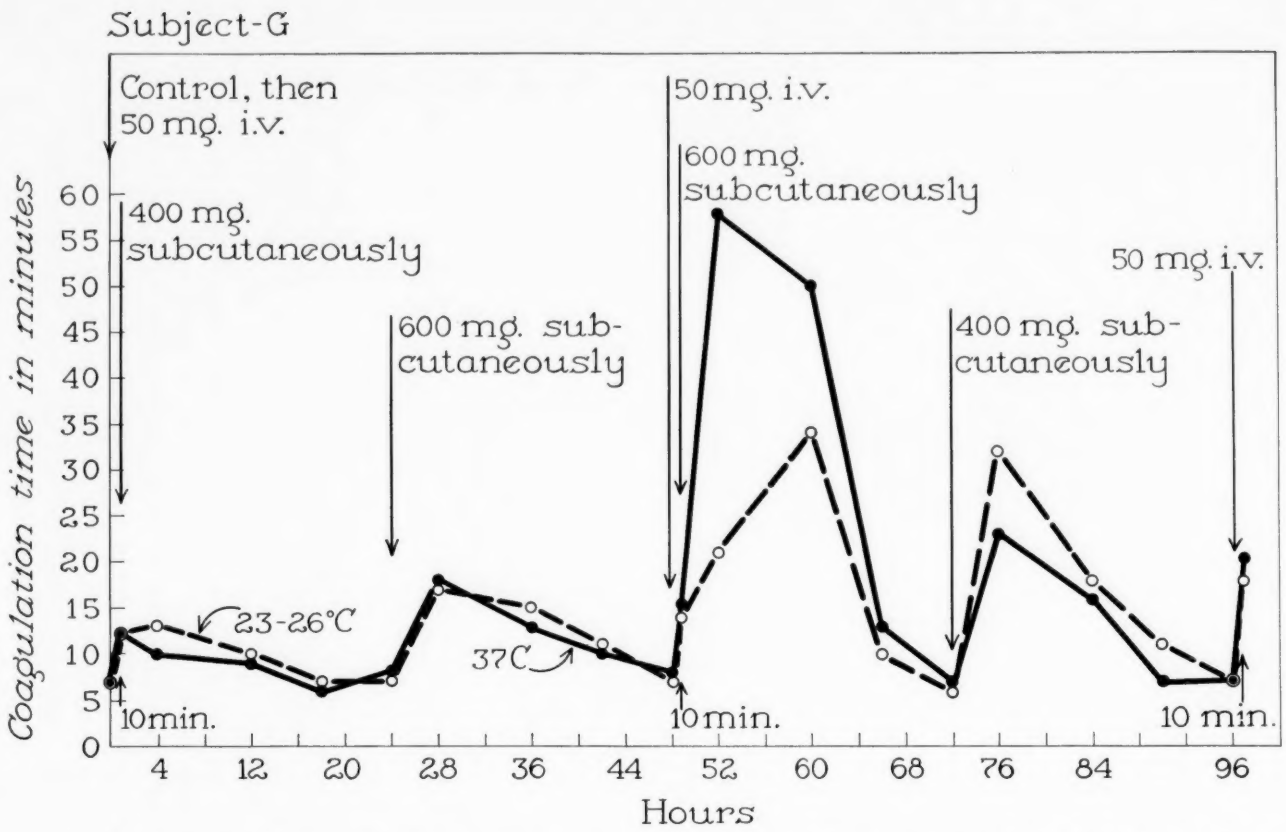

Fig. 2.—Response of coagulation time to heparin administered intravenously and subcutaneously to a man who had had an acute myocardial infarction. Although after forty-eight hours there was only a slightly increased response to heparin administered intravenously, there was a markedly increased response to heparin given subcutaneously as compared with the negligible response observed during the first two days.

Of the eleven periods studied in subjects who received a subcutaneous injection of 400 mg. of heparin, the coagulation time had returned to normal in twenty-four hours in eight. Adequate prolongation of the coagulation time continued for eighteen hours or longer in only five periods, and excessive increase in the coagulation time (greater than thirty minutes) was present at the end of four hours in seven.

Of the fourteen periods studied in subjects who received a subcutaneous injection of 600 mg. of heparin, the coagulation time had returned to normal in twenty-four hours in nine. Adequate prolongation of the coagulation time lasted for eighteen hours or longer in eight periods, and excessive increase of the coagulation time was present at the end of four hours in nine.

Characteristic responses to heparin encountered in this study are illustrated in Figs. 1 and 2. Spike and plateau types of curves were obtained with either 400 or 600 mg. of heparin given subcutaneously, even though subjects given each dose had similar initial responses to heparin administered intravenously. Furthermore, the subject showing a greater intensity of effect (L in Fig. 1) did not show a greater duration of effect.

At the completion of some of the studies, the heparin tolerance test was repeated (Figs. 1 and 2). Except for subject G (Fig. 2), little change in heparin tolerance was found according to the determinations of coagulation time made at 37° C.

Varying degrees of discomfort at the site of subcutaneous injection of heparin developed in more than half the subjects. Severe pain and marked local tenderness were experienced by normal subject B and patient G. Injections in the upper part of the arms were not as well tolerated as those in the gluteal regions. No subject refused subsequent injections. Hematomas developed at the injection site in two subjects.

SUMMARY

A study has been made of the effects in twenty human subjects of heparin given intravenously in 50 mg. doses and heparin given subcutaneously in a slowly absorbed medium in 200 mg., 400 mg. and 600 mg. doses. We are aware of the fact that the series of subjects is small and that the method of measuring the effect (coagulation time of whole venous blood) is subject to errors and inconsistencies. However, it appears from this study that the degree of prolongation of coagulation time following intravenously administered heparin cannot be used to predict the degree and duration of effect of a fixed dose of heparin given subcutaneously. In this study the duration of what we consider to be adequate prolongation of coagulation time following a dose of 400 or 600 mg. of heparin given subcutaneously in a slowly absorbed medium was usually less than twenty-four hours and excessive prolongation of coagulation time occurred in several instances following both doses. The unpredictable variability of degree and duration of effect of the heparin administered subcutaneously in a slowly absorbed medium indicates that if heparin is given

by this method an adequate, continuous and not excessive antithrombotic effect can be achieved only by individualization of dosage for each patient on the basis of frequent coagulation tests during the period of heparinization.

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A COMPARISON OF DIFFERENTIAL MEASUREMENTS OF SKIN TEMPERATURE USING A RADIOMETER, RESISTANCE THERMOMETER, AND THERMOCOUPLES

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SKIN-TEMPERATURE measurements have provided a valuable aid to the diagnosis and treatment of peripheral vascular disorders. The difference in the skin temperatures of corresponding points located on two limbs is of particular interest in many such cases. In cooperation with the National Naval Medical Center in Bethesda, Maryland, the National Bureau of Standards has made a series of comparison tests of some skin-temperature measuring instruments.

MATERIALS AND METHODS

Three types of instruments were used: a radiometer (1), a skin resistance thermometer (2) which consists of a resistance wire stretched across the end of a plastic tube, and differential thermocouples. All measurements were made under ordinary hospital ward conditions on patients who maintained a reclining position at least thirty minutes prior to and throughout the test. Both the radiometer and the skin resistance thermometer are commercially available and were unaltered. The four differential thermocouples were constructed of standard copper and constantan thermocouple wires. One wire at each junction was allowed to extend beyond the junction to facilitate fastening to the skin. The thermocouples were individually calibrated by placing the junctions in circulating water baths at known temperatures. A schematic diagram of the circuit used to measure the electromotive forces of the thermocouples is shown in Fig. 1.

The reliability of the Hardy Dermal Radiometer as a surface-temperature measuring instrument has been demonstrated by Stoll and Hardy[3] and this instrument was used as a

Table I. Average Deviations From Radiometer Readings in Degrees Centigrade

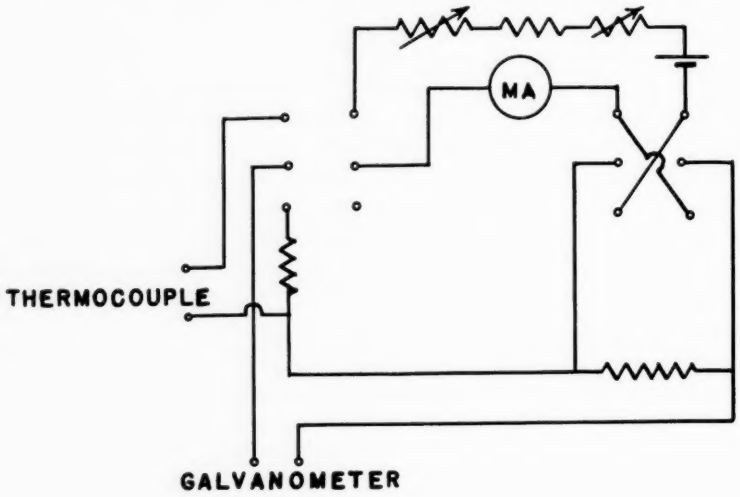
THERMOCOUPLES	RESISTANCE THERMOMETER
−0.06	+0.11
+0.03	−0.67
+0.28	−0.28
−0.13	−0.39
−0.18	−0.11
+0.01	−0.21
+0.17	−0.15
+0.01	−0.54
0	−0.09
+0.08	−0.36
+0.11	−0.93
+0.10	−0.25
−0.22	−0.26
−0.02	+0.03
+0.13	+0.43

From the Temperature Measurements Section of the National Bureau of Standards.
Received for publication May 7, 1951.

standard for these measurements. The method used in making the measurements of the temperature difference between corresponding points (for example, on the left and right feet) on the patient was to take alternate measurements of the temperature difference as shown by the radiometer, the resistance thermometer, and the thermocouples.

RESULTS

The results of the measurements described above were plotted as a function of time, and the average differences between the radiometer as a standard and the other two instruments were determined at one-minute intervals. In all



CIRCUIT FOR MEASURING THERMOCOUPLE EMF

Fig. 1.

TABLE II. AVERAGE DEVIATIONS OF THERMOCOUPLE READINGS FROM RADIOMETER READINGS IN DEGREES CENTIGRADE

WIRE SIZE	JUNCTIONS COVERED WITH SMALL PIECE OF TAPE	JUNCTIONS GLUED TO SKIN WITH DUCO CEMENT	JUNCTIONS BARE (FASTENED ADJACENT TO JUNCTION)
36 gauge	−0.16	−0.01	−0.02
	−0.09	−0.13	+0.13
	−0.09	−0.06	
	+0.23	+0.03	
	−0.03		
	−0.09		
	+0.22		
	+0.10		
	+0.02		
	−0.24		
	+0.12		
	+0.20		
	+0.30		
	0		
28 gauge	+0.08	+0.28	0
	−0.01	−0.13	+0.11
	+0.01	−0.18	+0.10
	+0.08	+0.01	−0.22
		+0.17	

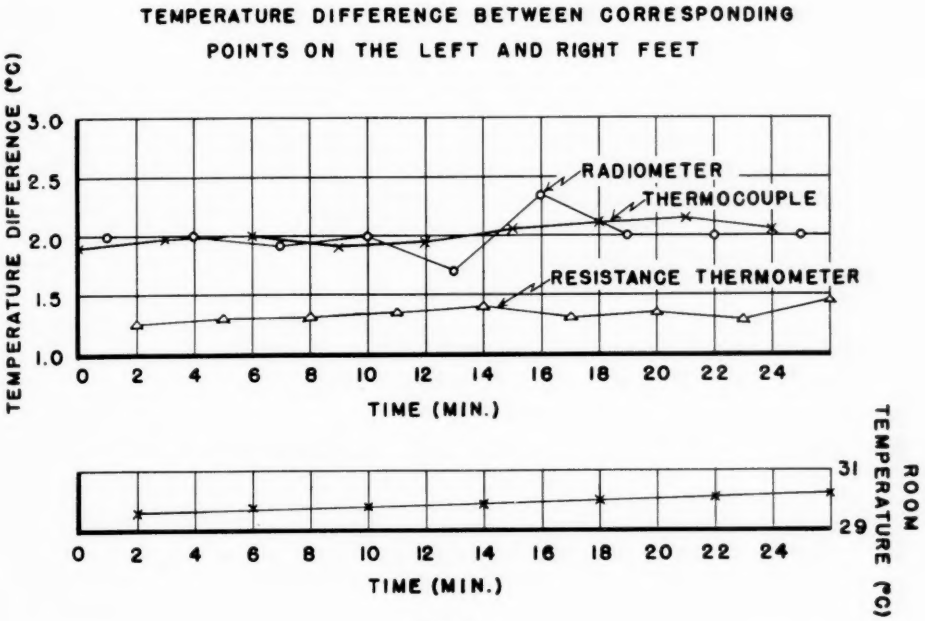

TEMPERATURE DIFFERENCE BETWEEN CORRESPONDING
POINTS ON THE LEFT AND RIGHT FEET
TEMPERATURE DIFFERENCE (°C)
3.0
2.5
2.0
1.5
1.0
RADIOMETER
THERMOCOUPLE
RESISTANCE THERMOMETER
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME (MIN.)
31
29
ROOM TEMPERATURE (°C)
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME (MIN.)

Fig. 2.

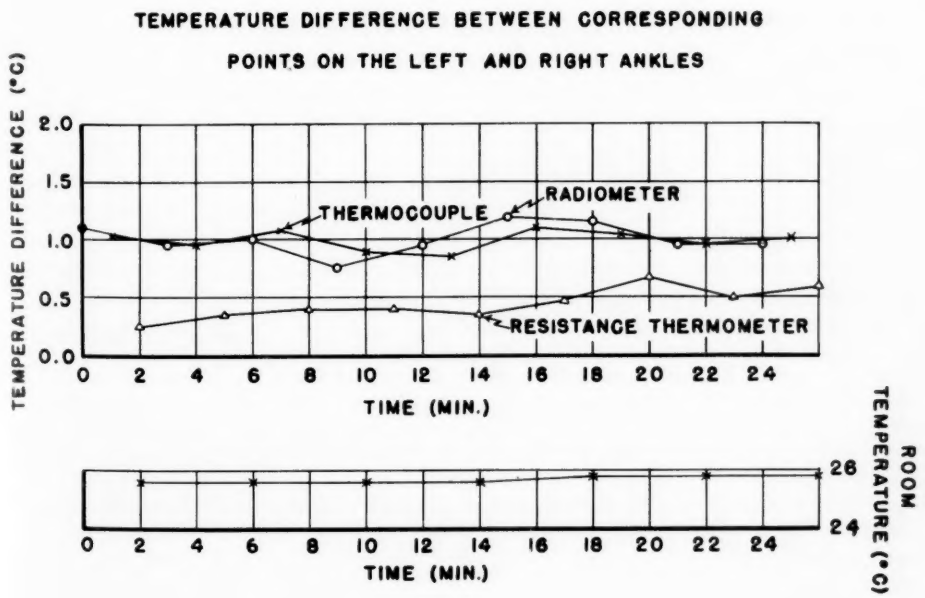

TEMPERATURE DIFFERENCE BETWEEN CORRESPONDING
POINTS ON THE LEFT AND RIGHT ANKLES
TEMPERATURE DIFFERENCE (°C)
2.0
1.5
1.0
0.5
0.0
THERMOCOUPLE
RADIOMETER
RESISTANCE THERMOMETER
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME (MIN.)
28
24
ROOM TEMPERATURE (°C)
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME (MIN.)

Fig. 3.

cases the thermocouple readings agreed with the radiometer readings to better than 0.3° C., as shown in Table I. No appreciable differences were noted in the results obtained with thermocouples of different wire sizes as indicated in Table II. Duco cement was found to be a satisfactory material to use in fastening the thermocouple junctions to the skin from the standpoint of allowing the patient limited freedom of motion without danger of pulling the junction away from the skin. The possibility of effects of wire sizes and misapplication of the thermocouples to the skin are well known.[4]

Examples of results for two different patients are shown in Figs. 2 and 3. It is apparent that the thermocouple readings agree closely with the radiometer readings, while those of the resistance thermometer are consistently low.

CONCLUSION

On the basis of these results, it is concluded that a comparatively simple arrangement of thermocouples may be used under normal conditions in hospitals to determine temperature differences with the same precision as that of the radiometer. By using two similar thermocouple junctions applied to the skin in the same maner, the effects of several variables including the method of fastening the junctions, the pressure which they exert, and the disturbance of the circulation in the region are considerably decreased.

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RIBOFLAVIN EXCRETION AS A FUNCTION OF PROTEIN METABOLISM IN THE NORMAL, CATABOLIC, AND DIABETIC HUMAN BEING

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THE theories which have been advocated to explain the negative nitrogen balance associated with the injury response can be classified into two general groups. The first maintains that there is an excessive breakdown of existing body proteins. The other maintains failure of the body to anabolize its proteins from ingested nitrogenous foods or circulating metabolites. The possibility of a combination of both mechanisms exists.

Several attempts have been made, by studying the simultaneous excretion of nitrogen, sulfur, potassium, and phosphorus, to test the hypothesis that negative nitrogen balance is due to the degradation of existing body proteins.[1] It was found that potassium and phosphate released from protein breakdown could become involved in the carbohydrate cycle. In addition, the phosphate could also be deposited in bone. The rate of excretion of these elements was therefore not a sole measure of their release from the breakdown of tissue protein.

It has been established that a flavin protein complex exists in the body in normal states. It has also been established that the concentration of this flavin protein complex decreases early in the course of protein starvation.[2, 3] The flavin component has been identified largely as riboflavin.

In the course of metabolic studies, on the vitamin requirements and utilizations of the diseased patient, simultaneous riboflavin and nitrogen excretions were measured. It was apparent that riboflavin excretion could be related to the positivity or negativity of the nitrogen balance. A more detailed investigation was undertaken to determine whether at least part of the increased nitrogen excretion in response to *injury* could be from the breakdown of existing flavin proteins in the body.

METHODS

The patients studied were hospitalized in individual rooms of the metabolic unit. They were permitted to eat only in their rooms. The diet prescribed was adjusted for the problem under study. All foods offered to the patients was weighed, and the rejected food was reweighed. The difference represented the food eaten. From this weighed intake all the nutrients studied were calculated by the use of standard tables.[4]

Twenty-four hour urine specimens were collected in amber colored glass bottles (0.75 ml. per cent glacial acetic acid was added as a preservative). The urines were kept re-

From the Metabolic Section of the Medical Services of the Mt. Sinai Hospital.
Aided by grants from the New York Foundation, Wakefield Fund, E. R. Squibb and Company, The American Dry Milk Institute, and the National Poultry and Egg Board.
Received for publication, June 6, 1951.
*Welt Fellow in Medicine.

TABLE I. SIMULTANEOUS NITROGEN BALANCE AND RIBOFLAVIN EXCRETION OBTAINED ON WELL-NOURISHED SUBJECTS EATING REGULAR HOSPITAL DIETS

METABOLIC PERIOD	J. K.		E. M.		F. W.		I. P.		H. S.		W. C.		E. S.		F. S.		A. H.	
	N_2†	% RIBO‡	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO
I*	-2.5	50.5	-1.9	22.7	+1.4	34.5	-2.5	52.0	+2.9	25.8	-1.2	69.0	+0.6	35.8	0.0	34.8	-2.1	57.5
II	+0.5	45.5	+4.3	16.6	-2.8	88.5	-1.0	35.6	+3.7	25.0	-1.5	50.0	+3.1	20.0	+1.6	32.2	-1.6	78.0
III	-1.6	56.0	+1.8	16.7	-3.5	72.0	+3.1	25.0	+3.9	30.0	+2.2	46.5	-0.6	39.0	-1.3	46.7	+6.6	31.4
IV	-0.4	64.0	+0.8	15.8	-0.1	46.0	-1.4	46.5	+3.1	26.2							-1.9	51.0
V	-2.0	53.0	-2.7	39.0	-4.6	64.0	-0.2	37.8										
VI	-2.0	58.7	-0.7	24.8	-0.8	27.0	-1.2	38.8										
VII	-1.0	47.1	-1.1	33.5	-0.1	20.0												
VIII	+0.2	49.5	-1.6	23.0														
IX	-2.0	77.2	-0.7	48.5														
X	-0.3	46.5																

*Roman numerals refer to metabolic periods average three days each.

†N_2 records nitrogen balance in grams for the period.

‡% Ribo records the percentage of the ingested riboflavin which appears in the urine.

frigerated at all times. In general the urines were pooled for the three-day metabolic periods indicated by the Roman numerals in the tables. Aliquots of the pooled urines were analyzed for nitrogen by the micro Kjeldahl method,[5] and riboflavin by the Ferrebee[6] method, as revised by Bessey and Lowry.[12] Nitrogen balances were constructed from these data assuming a constant fecal nitrogen of 1.3 Gm. per day.[7] Variations of ± 1.5 Gm. of nitrogen in equilibrium were not considered significant.

RESULTS

Control Group.—The normal range of riboflavin and nitrogen was established for the hospital "full diet" on eight well-nourished patients for a period of eleven to thirty days (average 17.7 days).

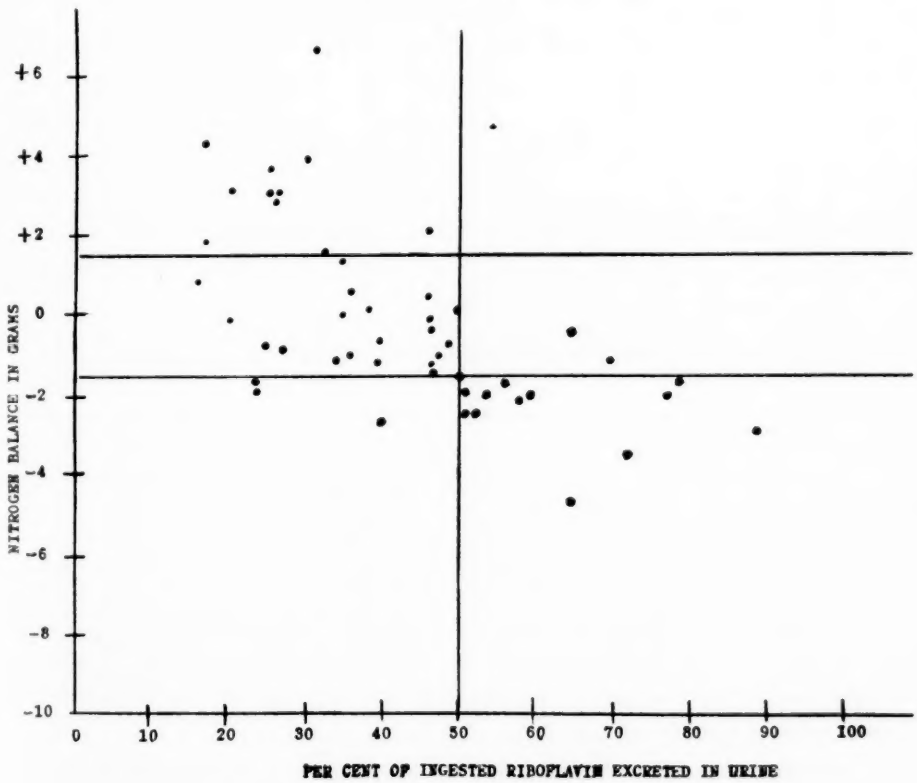

Fig. 1.

These patients subsisted on the routine hospital diet during which time they consumed an average of 2,073 calories, 13 Gm. of nitrogen, and 2,093 gammas of riboflavin per day.

The findings in this group are in agreement with those of Oldham.[8] Patients in caloric and nitrogen balance usually excreted less than 50 per cent of the ingested riboflavin in the urine. Patients in negative nitrogen balance almost always excreted more than 50 per cent of the ingested riboflavin.

The results are summarized in Tables I and II, and Fig. 1.

TABLE II. DISTRIBUTION ANALYSIS OF DATA PRESENTED IN TABLE I.

RIBOFLAVIN EXCRETION	NITROGEN BALANCE		
	POSITIVE +1.5 GM./DAY	EQUILIBRIUM +1.5 to −1.5 GM./DAY	NEGATIVE −1.5 GM./DAY
Under 50%	12	21	2
Over 50%	0	2	12

TABLE III. SIMULTANEOUS NITROGEN BALANCE AND RIBOFLAVIN EXCRETION OBTAINED ON A GROUP OF POSTOPERATIVE AND CONVALESCENT SURGICAL PATIENTS

METABOLIC PERIOD	J. L.		H. W.		J. M.		M. F.		J. C.		H. H.	
	N_2†	% RIBO‡	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO	N_2	% RIBO
I	–	I-0.0 E-190.0	−5.7	350.0	−5.0	765.0	−0.4	36.0	+9.4	29.0	+2.4	29.0
II	−11.2	I-0.0 E-740.0	−1.9	28.0	−4.0	36.0	–	133.0	+4.0	35.0	+5.4	41.0
III	−11.9	I-0.0 E-505.0	−0.6	8.5	+1.1	50.0	–	23.0	+6.7	25.0	+7.3	52.0
IV	−3.0	80.0	+2.3	9.8			6.6	112.0*	–	–	+5.1	50.0
V	+1.0	68.0	+2.5	24.0			+0.8	78.0*	+3.2	23.0	+7.2	41.0
VI	−0.3	–					0.0	115.0*			+6.3**	56.0
VII	−3.4						+1.6	45.0*			+15.3**	62.0
VIII	0.0						+9.2	51.0*			+12.8**	94.0
IX	+2.1	42.0					+11.8	39.0			+15.1**	86.0
X											−19.8**	120.0

Roman numerals refer to metabolic periods average three days each.
*6.0 mgs supplementary riboflavin by mouth daily.
**Saturation phenomena observed in convalescent patient.
†N_2 records nitrogen balance in grams for the period.
‡% Ribo records the percentage of the ingested riboflavin which appears in the urine.

TABLE IV. DISTRIBUTION ANALYSIS OF DATA PRESENTED IN TABLE III.

RIBOFLAVIN EXCRETION	NITROGEN BALANCE		
	POSITIVE +1.5 GM/DAY	EQUILIBRIUM +1.5 TO −1.5 GM/DAY	NEGATIVE −1.5 GM/DAY
Under 50%	13	4	2
Over 50%	2(5)*	3	6

*Saturation phenomena observed in convalescent patient, H. H.

Surgical and Convalescent Group.—The next group studied in this manner was composed of postoperative and convalescent patients. Six of these were studied on the metabolism ward. Results of these findings are presented in Tables III and IV, and Fig. 2.

Patient J. L. was a 47-year-old white man with a twelve-year history of duodenal ulcer. His preoperative nutritional status was good. He was operated upon at metabolic period I (one day) when subtotal gastrectomy, splenectomy, and bilateral infradiaphragmatic vagotomy were performed. During metabolic periods II and III (three and two days, respectively) with no riboflavin intake, he excreted an average of 740 gammas for the first three days and 505 gammas for the next two days, representing a total negative balance of 3,230 gammas. As his nitrogen balance equilibrated and became positive, his riboflavin retention increased to normal values.

H. W., on whom a cholecystectomy was done, was negative 5.7 Gm. of nitrogen on the day of operation (metabolic period I) and excreted 3.5 times

as much riboflavin as he ingested. In the next three days (metabolic period II) his nitrogen balance averaged 1.9 Gm. per day negative. Subsequently he was in nitrogen equilibrium or positive balance, and the riboflavin retentions were above average.

J. M. underwent herniorrhaphy. On the day of operation (metabolic period I) he excreted 7.65 times his riboflavin intake. In spite of a three-day (period II) delay in return to nitrogen equilibrium his percent riboflavin excretion dropped markedly.

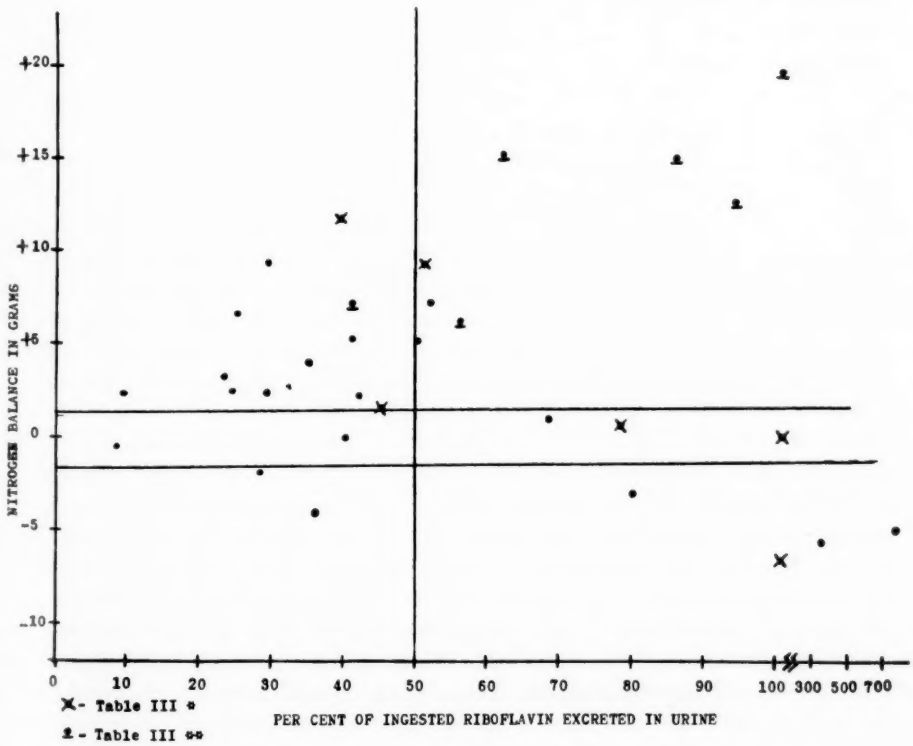

Fig. 2.

M. F. had a one-year history of cough with an 18 pound weight loss. Studies revealed a carcinoma of the right upper lobe of the lung. Period I represents a four-day preoperative study showing nitrogen equilibrium and 36 per cent excretion of the ingested riboflavin. The day of operation (period II) the patient had a nitrogen excretion of 6.2 Gm., but received 1,500 c.c. of whole blood in addition to intravenous glucose. In view of the uncertainty of the rate of metabolic availability of the blood proteins, nitrogen balance figures cannot be constructed for this period and the subsequent four days (period III). In metabolic period VI when the patient was in negative nitrogen balance and for the remainder of his hospital stay the patient was given 6 mg. of riboflavin

postoperatively in addition to that contained in his food. It is to be noted that his ability to retain even this amount of riboflavin was very poor until the nitrogen balance became markedly positive.

The last two patients in this group were admitted to the metabolism ward for nutritional rehabilitation following prolonged periods of hospitalization and several surgical procedures.

Patient H. H. was admitted for thrombocytopenic purpura of undetermined etiology. During a period of six months on the surgical service she successively underwent splenectomy, drainage of a subphrenic abscess, drainage of a left empyema, and decortication of the left lung. In this time her weight decreased from 123 to 88 pounds. She was given large amounts of various vitamins orally and parenterally while on the surgical service. One month after her last operation she was admitted to the metabolism ward. For the first eighteen days (periods I to VI) she consumed a diet averaging 2,659 calories and 15.9 Gm. of nitrogen daily. She gained 4.5 kg. in body weight without visible edema. Her intake was then increased for the next twelve days (periods VII to X) to an average of 3,393 calories and 32.5 Gm. of nitrogen. She gained an additional 4.2 kg. in weight. It is to be noted that in spite of the large amounts of vitamins administered during her stay on the surgical wards with an inadequate caloric intake, as reflected by her weight loss, she was not saturated with respect to these nutrients and was able to retain riboflavin efficiently as soon as her nitrogen balance became consistently positive. A saturation phenomenon was observed later during her period of hyperalimentation (periods VII to X).

Patient J. C. had had a draining chest sinus for five years following the removal of a supposed echinococcus cyst of the liver at another hosiptal. While on the surgical service in this hospital he had two thoracotomies and a muscle flap implantation. He gained one pound in his two months on the surgical service. At the time of his transfer to the metabolism ward, one month after the last surgical procedure, the patient was anorexic, listless, and underweight. He had received vitamins by mouth and parenterally. His caloric intake had been just adequate to maintain his weight. When a diet averaging 2,800 calories and 20.5 Gm. of nitrogen was given, this patient gained weight, remained in positive nitrogen balance, and showed an excellent ability to retain his ingested riboflavin.

The postoperative group studied under these conditions demonstrates that in the immediate postoperative period when the nitrogen balance is maximally negative, the riboflavin excretion exceeds the intake. As nitrogen equilibrium is approached and positive nitrogen balance is achieved, the per cent of riboflavin excreted drops markedly. In the convalescent patients, who were in poor nutritional status at the onset of these observations, a positive nitrogen balance is associated with high riboflavin retention.

It is to be noted in patient H. H. that a saturation phenomenon is found after a period of forced protein riboflavin feeding (vide infra).

Diabetic Group.—There are many limitations to the use of postoperative patients in studying these injury response mechanisms. The increased nitrogen excretion is usually of limited duration. Its tendency to spontaneous improve-

ment is uncontrollable. The therapeutic demands during the critical postoperative time take precedence over the strict experimental conditions that are desired, and thus time is wasted when patients are lost from the study group.

It became apparent then that if a group of patients could be found in whom the injury response could be reversed and metabolic equilibrium achieved at will, they could be studied to greater advantage. Such patients could serve as their own controls. The patient with clinical diabetes mellitus theoretically should lend himself to this type of reversibility. It is known that insulin withdrawal will result in glycosuria and negative nitrogen balance in many patients with the diabetic syndrome.[10]

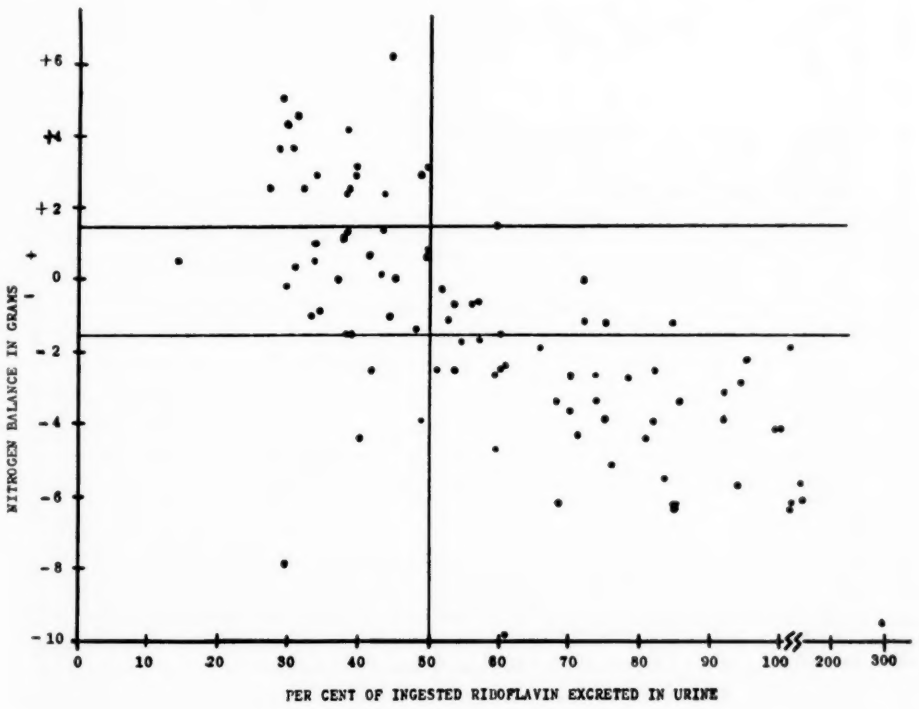

Fig. 3.

A third study group of patients with known diabetes mellitus was admitted. Eight of these were diabetics of three to twenty-four years' duration and had been under observation in the outpatient department for most of the duration of their disease.

This group was studied in the metabolism ward under the same general conditions as the other two groups, except that attempts were made to vary nitrogen balance at will by the administration or graded withdrawal of insulin. These variations in insulin administration are indicated in general by the variations in glycosuria and the degree of negativity of the nitrogen balance. No attempt will be made to report the complete data collected at this time.

TABLE V. SIMULTANEOUS NITROGEN BALANCE, RIBOFLAVIN AND GLUCOSE EXCRETIONS OBTAINED IN A GROUP OF DIABETICS WHOSE GLYCOSURIAS ARE USUALLY DIFFICULT TO CONTROL

METABOLIC	H. L.			O. B.			J. S.			A. S.		
PERIOD	N_2†	% RIBO‡	GLUCOSE§	N_2	% RIBO	GLUCOSE	N_2	% RIBO	GLUCOSE	N_2	% RIBO	GLUCOSE
I*	+0.2	42.7	31.0	-1.5	38.0	-	-5.7	136.0	19.0	+1.0	33.4	27.0
II	-4.3	71.4	106.0	-0.8	34.0	-	-8.3	-	83.0	+3.1	49.4	27.0
III	-3.4	67.7	93.0	-2.6	59.0	-	-3.1	92.0	31.0	-1.1	72.0	109.0
IV	-5.5	83.2	109.0	+2.5	32.0	-	-5.1	76.0	48.0	0.0	72.0	118.0
V	-4.4	40.0	78.0	+0.5	14.1	2.0	-4.1	100.0	70.0	0.0	45.0	144.0
VI	-1.6	57.0	40.0	-1.0	32.7	0.0	-2.2	95.0	8.0	+3.7	30.2	66.0
VII	-9.8	61.3	44.0	-3.9	82.2	106.0	-1.8	118.0	8.0	+3.6	28.4	45.0
VIII	-2.6	73.4	42.0	-6.2	122.0	164.0	-2.6	70.0	38.0	+1.2	37.5	20.0
IX	-1.2	84.0	43.0	-2.5	82.2	43.0	-3.6	70.0	21.0	+1.3	37.7	19.0
X	-3.4	85.5	64.0	-2.5	50.7	6.0	-2.8	94.0	79.0	+0.8	49.7	95.0
XI	-1.3	47.9	28.0	+0.3	30.7	5.0	-0.6	57.0	44.0	+0.7	41.7	74.0
XII	-1.8	66.0	43.0	-0.2	29.7	29.0	-5.8	85.0	93.0	+2.9	39.5	60.0
XIII	-2.5	60.0	84.0	-3.9	48.7	97.0	-2.7	78.0	102.0	+2.9	33.8	89.0
XIV	-1.5	37.8	94.0	0.0	37.0	137.0	-6.1	138.0	98.0	+3.1	39.5	17.0
XV	-1.5	60.0	106.0	+2.5	27.4	117.0	-4.1	99.0	108.0			
XVI	-2.5	53.2	119.0	+2.5	38.1	77.0	-5.7	94.0	106.0			
XVII	-3.8	75.0	132.0	+2.4	43.5	74.0	-4.4	81.0	95.0			
XVIII	+2.9	48.5	41.0	+1.4	43.1	89.0	-3.8	92.0	141.0			
XIX	+0.5	33.3	31.0	+2.4	37.7	67.0	-6.4	85.0	137.0			
XX	-1.7	54.5	44.0	+5.0	28.9	100.0	-9.5	296.0	186.0			
XXI	-0.7	56.1	19.0	-7.8	29.6	121.0						
XXII	-3.3	73.7	25.0	+4.2	37.7	92.0						
XXIII	-1.0	44.5	42.0	+4.3	29.9	87.0						
XXIV	-2.5	41.8	44.0	+4.5	31.6	56.0						
XXV	-4.7	59.3	79.0	+6.2	44.4	9.0						
XXVI	-6.2	68.5	127.0	+1.5	59.4	64.0						
XXVII	-2.4	60.5	120.0									
XXVIII	-1.1	52.5	144.0									
XXIX	-6.4	116.0	111.0									
XXX	-1.2	75.5	49.0									
XXXI	-0.7	53.1	115.0									
XXXII	-0.3	51.9	53.0									
XXXIII	+0.7	49.7	0.0									

*Roman numerals refer to metabolic periods average three days each.

†N_2 records nitrogen balance in grams for the period.

‡% Ribo records the percentage of the injested riboflavin which appears in the urine.

§Glucose expressed as average excretion of grams daily in each metabolic period.

Patients O. B., J. S., H. L., and A. S. were selected because they were considered severe both from the point of insulin requirement and difficulty of control of glycosuria (Tables V and VI, and Fig. 3). The first three of these were well-nourished adults in whom marked negativity of nitrogen balance could be induced by the withdrawal of insulin. The fourth patient (A. S.) was an undernourished 14-year-old girl who had had several recent admissions for ketotic acidosis. Failure to achieve marked negative nitrogen balance in this patient is possibly based on the same mechanism as is frequently observed in undernourished individuals who fail to manifest the so-called injury response.

TABLE VI. DISTRIBUTION ANALYSIS OF DATA PRESENTED IN TABLE V

	NITROGEN BALANCE		
RIBOFLAVIN EXCRETION	POSITIVE +1.5 GM./DAY	EQUILIBRIUM +1.5 TO -1.5 GM./DAY	NEGATIVE -1.5 GM./DAY
Under 50%	17	20	3
Over 50%	0	12	36

The same general correlation of negative nitrogen balance with increase in riboflavin excretion was also found in this group. Excretions of more riboflavin than was ingested were found when the nitrogen balance became markedly negative. This can be compared in principle with the postoperative patients. It is realized that the riboflavin intake of the postoperative patients was at times minimal, while that of the diabetic was kept at a constant average normal level of approximately 2 mg. daily.

In the patient O. B. a variation of the conditions was introduced to demonstrate that this riboflavin excretion is not a function of the glycosuria or polyuria, but rather of nitrogen balance. In periods XV to XXIII forced protein feedings were given, and sufficient insulin administered to maintain nitrogen balance and prevent ketosis, but not to inhibit glycosuria. It is to be noted that while the patient remained in positive nitrogen balance[11] in spite of polyuria, and glycosuria equal to about 50 per cent of his carbohydrate intake, the riboflavin excretions indicate a good retention of this vitamin. O. B., while in mild ketosis (VIII), excreted more riboflavin than he ingested. During his replenishment periods (XV-XXVI) his riboflavin retention was excellent despite marked glycosuria. The glycosuria at this particular time represents not so much insulin withdrawal as increased carbohydrate intake, since the insulin administration was constant at 70 units daily, while the diet intake had been increased.

J. S. demonstrated this inability to retain riboflavin in his period of negative nitrogen balance, and in the XX metabolic period with maximum glycosuria and nitrogen negativity riboflavin excretion was greater than that ingested.

H. L. followed the pattern established by O. B and J. S.

In the case of A. S., the undernourished individual, a consistently significant negative nitrogen balance was impossible to provoke in spite of the degree of glycosuria achieved. The riboflavin retention was excellent during the periods of nitrogen retention, and decreased slightly with the periods of minimal nitrogen negativity.

An additional group of obese diabetics who consumed caloric intakes below maintenance levels showed consistently negative nitrogen balances (Tables VII and VIII, and Fig. 4). In very few of the metabolic periods did they fail to show the same relationship between negative nitrogen balance and riboflavin excretion.

It is admittedly hazardous to draw conclusions from data on diabetic patients, since they represent metabolic anomalies. Certainly the problem of gluconeogenesis from protein is a complicating factor in the evaluation of the data.

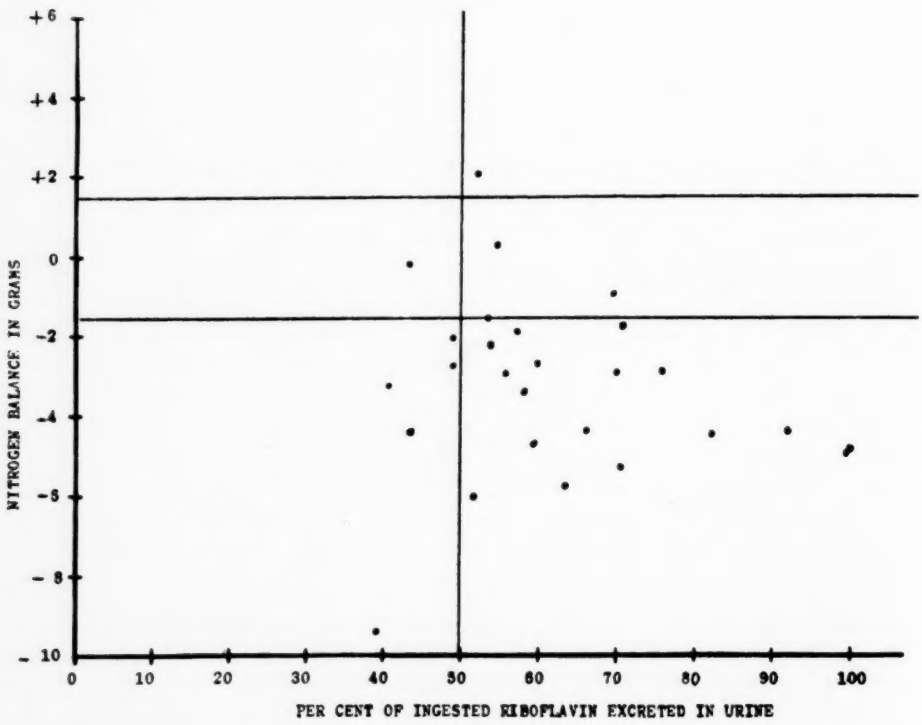

Fig. 4.

In view of the similarity of the findings in the diabetic as well as the nondiabetic groups, it may be assumed that these phenomena must have metabolic aspects in common. Insulin withdrawal can be likened to a true catabolic phase with breakdown of the flavoproteins.

DISCUSSION

The main point requiring clarification is whether negative nitrogen balance is due to excessive breakdown of existing protein or represents failure of anabolism including failure to utilize ingested protein. It is fair to assume for purposes of discussion that when the riboflavin excretion is in excess of that ingested, this surplus must be derived from stored riboflavin in the body. Since

it is known that free riboflavin exists only in minute quantities, and that the bulk of the body riboflavin is combined with protein, one can assume that any appreciable excess quantities of riboflavin in the urine must be derived from the breakdown of the flavoproteins. Therefore the negative nitrogen balance of the catabolic phase can be considered a breakdown of at least one group of body proteins.

Where negative nitrogen balance exists because of caloric deficit or other noninjury response phenomena, there is apparently some loss of ability to retain riboflavin, but there is no excretion in excess of intake. This would indicate that either there is a failure of synthesis of flavoproteins, or synthesis is normal, but there is an increased breakdown of existing proteins to upset the equilibrium.

The saturation phenomenon observed during the period of forced protein and riboflavin intake raises an interesting point for speculation. The establishment of an over-all nitrogen balance indicates that there is an eventual equilibrium between all proteins in the body. The rates of achievement of the equilibria of the various protein systems are most likely variable. One can apply to this problem the concept of variability in the lability of proteins as advocated by Spinson and Rittenberg.[9] Fig. 5 expresses diagrammatically a system into which many of the reported observations will fit.

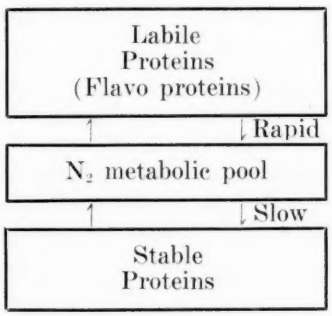

Fig. 5.

TABLE VII. SIMULTANEOUS NITROGEN BALANCE, RIBOFLAVIN AND GLUCOSE EXCRETIONS OBTAINED IN A GROUP OF OBESE DIABETICS SUBJECTED TO INADEQUATE CALORIC INTAKE

METABOLIC PERIOD	I. P.			S. S.			M. R.			G. D.		
	N_2†	% RIBO‡	GLUCOSE§	N_2	% RIBO	GLUCOSE	N_2	% RIBO	GLUCOSE	N_2	% RIBO	GLUCOSE
I*	-3.2	40.6	0.0	-2.9	55.7	12.0	-1.5	53.1	30.0	-4.8	100.0	31.0
II	-5.2	70.5	8.0	-6.0	51.5	23.0	-4.3	66.4	42.0	-2.8	76.2	16.0
III	-4.9	99.4	34.0	-5.7	63.0	9.0	-1.8	57.4	33.0	+0.4	54.5	3.0
IV	-2.8	70.0	52.0	-4.6	58.5	25.0	+2.1	52.4	14.0	-1.7	70.8	0.0
V	-4.4	82.4	53.0	-2.7	48.6	35.0	-0.9	69.7	5.0			
VI	-2.6	59.7	46.0	-9.4	38.5	17.0	-0.2	43.0	11.0			
VII	-3.4	57.8	33.0	-2.0	48.0	3.0						
VIII	-2.2	53.6	8.0									
IX	-4.3	72.3	3.0									
X	-4.4	43.1	0.0									

*Roman numerals refer to metabolic periods average three days each.
†N_2 records nitrogen balance in grams for the period.
‡% Ribo records the percentage of the ingested riboflavin which appears in the urine.
§Glucose expressed as average excretion of grams daily in each metabolic period.

TABLE VIII. DISTRIBUTION ANALYSIS OF DATA PRESENTED IN TABLE VII

RIBOFLAVIN EXCRETION	NITROGEN BALANCE		
	POSITIVE +1.5 GM./DAY	EQUILIBRIUM +1.5 TO -1.5 GM./DAY	NEGATIVE -1.5 GM./DAY
Under 50%	0	1	4
Over 50%	1	3	19

One may postulate a nitrogen metabolic pool of amino acids and other protein metabolites. This pool probably is in equilibrium with all the protein systems in the body. The evidence is in favor of a whole spectrum of protein labilities. For purposes of this discussion in Fig. V these are schematically represented as (a) the very labile group of proteins of which the flavo proteins are one, and (b) the relatively stable protein system.

There are no large stores of free amino acids in the body. Their presence would upset the homeostatic mechanisms. If any or all proteins were called upon to supply these amino acids in times of stress, there would be interference with the basic functional and morphologic structures of the body. The labile proteins in times of metabolic stress are available for very rapid degradation to supply these protein metabolic units when needed, without interfering with any other functions or structure.

Re-equilibration between the stable proteins and the labile reserve takes place slowly through the metabolic pool. Ingested protein is used to refill the reservoirs. It is conceivable that after prolonged periods of protein losses the labile reserves are exhausted and some depletion of stable protein has resulted. This is manifested by the failure of the "protein catabolic response" in the depleted or undernourished individual and the eventual muscle wasting that occurs. The reverse may also hold true. The labile reserves can be replenished long before the stable proteins are replaced. Thus it is possible to have a high riboflavin retention during one phase of positive nitrogen balance and a normal or poor riboflavin retention in the subsequent period of positive nitrogen balance. This can be one possible explanation for the saturation phenomenon observed in patient H. H.

The flavo proteins may represent only one of the labile proteins, and it can be assumed that there are other proteins whose breakdown will contribute to the urinary nitrogen excretion. One therefore cannot expect to find a consistent fixed ratio between the net nitrogen gain or loss and riboflavin excretion, under the conditions of these experiments.

These findings raise the question as to the validity of the current practice of assaying the nutritional status of a single individual with respect to riboflavin by means of urinary excretions or load tests. It is demonstrated here that the percentage excretion of riboflavin can be related to the current and immediately past nitrogen balance. In mass surveys under most conditions, individual variations are averaged out, and urinary excretion data can be of considerable value.

CONCLUSIONS

Evidence is presented that nutritionally normal individuals will usually retain more than 50 per cent of the ingested riboflavin when in nitrogen equilibrium, and less than 50 per cent when in negative balance.

Intense negativity of nitrogen balance precipitated by surgical procedures is associated with an excretion of riboflavin many times greater than the ingested amounts. In the postoperative convalescent periods as the nitrogen balance equilibrates and becomes positive, riboflavin retention increases to normal or greater than average normal levels.

Negative nitrogen balance induced in the diabetic by withholding part of his insulin requirements or by caloric restriction results in an excretion of more than 50 per cent of the ingested riboflavin.

Intense negativity of nitrogen balance precipitated by insulin withdrawal in the diabetic patient is associated with an excretion of riboflavin many times greater than the ingested amounts. Nitrogen equilibrium and positive nitrogen balances are associated with normal riboflavin excretions even in the presence of marked glycosuria.

The significance of these findings is discussed in relationship to catabolic phenomena and the interpretation of riboflavin load tests and urinary excretion data.

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SODIUM AND POTASSIUM CONCENTRATION IN HUMAN CEREBROSPINAL FLUID

I. Normal Values

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THE role of electrolytes in the maintenance of normal equilibrium of body fluids has been the subject of numerous recent studies. The introduction of the flame photometer has made determination of electrolyte concentrations more feasible and, no doubt, offers one reason for the recent renewed interest in electrolyte metabolism.

Increased interest has been displayed in the concentration of potassium and sodium in the blood serum, but relatively little interest has been displayed concerning the role of these electrolytes in the fluids of body cavities, principally that of the cerebrospinal fluid. Previous studies on the concentration of these elements in the cerebrospinal fluid have been confined to a relatively small series of cases. In the eight published reports reviewed, the values reported were determined from a study of from three to twenty cases. It is obvious that a study of a larger series of cases is indicated.

MATERIAL FOR STUDY

One hundred cerebrospinal fluid specimens of apparently normal individuals were obtained from patients admitted to the Jefferson-Hillman Hospital. No attempt was made to evaluate changes in the sodium and potassium concentrations as it pertained to age, race, or sex. One hundred consecutive patients were examined.

All obvious diseases, such as congestive heart failure, hypertension, or renal diseases, were excluded. Patients with obvious involvement of meninges, such as meningitis, were excluded. All cases of syphilis that were shown by cerebrospinal fluid findings to involve the central nervous system were not included in this study.

TECHNIQUE

The Beckman flame photometer was employed, using a slightly modified procedure originally described by Mosher and associates.[1] In order to adapt the procedure to the examination of cerebrospinal fluid, the standard was modified so that it would closely approximate that of cerebrospinal fluid. The following standard solution was used.

Na	143.5 meq.	8.3880 Gm. NaCl/liter
K	3.5 meq.	0.2610 Gm. KCL/liter
P	2.35 meq.	0.2704 Gm. $NH_4H_2PO_4$/liter
Ca	5.0 meq.	0.2777 Gm. $CaCl_2$/liter
Mg	1.5 meq.	0.1523 Gm. $MgCl_2$—6 H_2O/liter
Glucose		0.7000 Gm. Glucose/liter
Urea		0.3000 Gm. Urea/liter

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Received for publication, May 17, 1951.

A portion of this work was made possible by a grant from Irwin, Neisler and Company, Decatur, Ill.

*In partial fulfillment of the requirements for the M.S. degree in Biochemistry.

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The gelatin and cholesterol were omitted from the standard solution, inasmuch as there are negligible amounts of protein and cholesterol in cerebrospinal fluid. One cubic centimeter of 95 per cent ethyl alcohol was added in the 1:50 dilution of both standard and cerebrospinal fluid specimen in order to reduce surface tension, thus permitting a more uniform rate of flow through the atomizer.

RESULTS

The findings upon examination of one hundred cerebrospinal fluid specimens are shown in Table I.

TABLE I*

	Na MEQ.	Na MG./100 C.C.	K MEQ.	K MG./100 C.C.
100 Specimens (mean)	142.47	327.5	2.88	11.80
Mean (10 highest values)	149.3	343.9	3.15	12.91
Mean (3 highest values)	151.3	348.0	3.35	13.72
Highest value	152.0	349.5	3.65	14.26
Lowest value	134.0	308.0	2.50	9.78
Mean (10 lowest values)	134.8	310.0	2.60	10.17
Mean (3 lowest values)	134.0	308.0	2.50	9.78

*Findings are expressed both as milligrams per 100 c.c. and as milli-equivalents.

In order to fully evaluate this study, comparative determinations were made on ten specimens, using established chemical procedures.[2, 3] These determinations are shown in Table II.

TABLE II

METHOD	Na MEQ.	Na MG./100 C.C.	K MEQ.	K MG./100 C.C.
Flame photometer	145.1	333.5	2.94	12.1
Chemical	143.8	330.5	3.13	12.82

The values obtained by both methods were found to be in close agreement, although slightly lower values for sodium and slightly higher values for potassium were obtained from the chemical method of analysis.

COMMENT

A review of the literature showed fairly good correlation of the cerebrospinal fluid concentrations of these elements. A comparative study of the reports reviewed are given in Tables III and IV.

TABLE III. A CORRELATION OF CEREBROSPINAL FLUID POTASSIUM LEVELS REPORTED BY OTHER INVESTIGATORS[4]

INVESTIGATOR	DATE	NO. OF CASES	K RANGE IN MG./100 C.C.	K AVERAGE MG./100 C.C.	K AVERAGE MEQ./LITER	METHOD OF ANALYSIS
Leubner et al.	1928	20	11-16	12.3	3.14	Gravimetric
Pincus & Kramer	1923	3	12.8-16.6	15.1	3.86	Gravimetric
Leipold	1928	16	10.5-16.9	13.5	3.47	Not given
Merritt & Smith	1937	15	11-15	13.2	3.38	Not given
Honeyman & Zwemer	1938	10	12-14.5	13.3	3.40	Not given
Eisler	1928	19	13-13.5	13.2	3.38	Gravimetric
Lierle & Sage	1931	4		12.8	3.27	Gravimetric
Duliere	1931	14		11.17	2.85	Gravimetric
Average of above cases				13.07	3.34	

TABLE IV. A CORRELATION OF CEREBROSPINAL FLUID SODIUM VALUES AS DETERMINED BY OTHER INVESTIGATORS[4]

INVESTIGATOR	DATE	NO. OF CASES	RANGE IN MG./100 C.C.	AVERAGE MG./100 C.C.	RANGE IN MEQ./LITER	METHOD OF ANALYSIS
Dailey, M. E.	1931		301-304		131-132	Not given
Shohl					151	Given as normal value
Sunderman & Boerner			312-352		135-153	Flame photometry

The potassium mean value (2.88 meq.) in Table I is lower than the mean of eight investigators (3.34 meq.), as shown in Table III. Such a discrepancy may be partially accounted for by the difference in the number of specimens examined. In Table I, the mean of ten highest values is 3.15 meq., while the mean of three highest values is 3.35 meq. By careful selection, almost identical findings of the investigators listed above may be found in our one hundred specimens, which indicates the hazard of presenting so-called normal values, based on a limited number of determinations.

Sodium values by most investigators closely parallel those of blood serum as referred to by Cantarow and Trumper.[5] Shohl in "Mineral Metabolism"[6] has reported 151 meq. per liter as normal sodium concentration in cerebrospinal fluid. Sunderman and Boerner[4] give a normal range of 135 to 153 meq. These latter figures were determined by flame photometry. These findings are in essential agreement with the values found in our series.

SUMMARY AND CONCLUSIONS

A study of the sodium and potassium concentration in the cerebrospinal fluid of one hundred apparently normal individuals has been presented. A correlation of the values obtained by the flame photometer and chemical method has been made.

The invaluable assistance and numerous suggestions of H. C. Elliott and Drs. Emmett B. Carmichael and W. W. Carlson, all of the Department of Biochemistry, Medical College and School of Dentistry, University of Alabama, is gratefully acknowledged.

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THE DIFFUSION OF DEXTRANS OF DIFFERENT MOLECULAR SIZES TO LYMPH AND URINE

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PARTIALLY hydrolyzed dextrans introduced in therapy for maintenance of adequate volume of circulating blood by Grönwall and Ingelman[1, 2] have received favorable confirmation from Lundy and his associates.[3-6] Many solutions of macromolecular substances have been used for this purpose because of osmotic activity in expanding the volume of plasma while the concentration of the injected material in the blood exceeded that of the extracellular fluid. Excretion rates of dextrans in the urine, largely dependent on molecular size, and the excretion of intravenously injected dextrans through the intestinal mucosa[7] indicate that these substances pass out of the blood. Plasma and urine have been found free of dextran within two or three weeks, although traces of the substance have been detected with serologic methods in lymphoid tissue, spleen, and kidney eight weeks after its administration.[8] No evidence of specific sequestration of dextran in appreciable amounts in any of the organs of the body has been found.[9] The mode of disposal of that which is not excreted in the urine remains in doubt. No sign of enzymatic breakdown was noted when this substance was incubated with blood or extracts or suspensions of tissue. It has not been found in the feces[10] or excreted in the bile, but as much as 28 per cent of the injected dextran has been found excreted into the alimentary canal within nine hours. It has been suggested that the polysaccharide is broken down to glucose and then metabolized.

TABLE I. DEXTRAN CONCENTRATION IN BLOOD AND LYMPH OF RATS

MOLECULAR WEIGHT OF DEXTRAN	CONCENTRATION, MG. PER 100 ML., AT VARYING TIMES AFTER INJECTION OF 300 MG. OF DEXTRAN							
	3 HOURS		6 HOURS		24 HOURS		72 HOURS	
	BLOOD	LYMPH	BLOOD	LYMPH	BLOOD	LYMPH	BLOOD	LYMPH
205,000	745	610	730	600	390	430	140	160
120,000	605	610	590	480	280	320	80	105
85,000	425	415	460	440	210	275	65	40
52,000	465	635	450	480	215	245	55	95
24,000	180	480	135	230	120	140	60	85

From these observations it is evident that dextrans diffuse rather rapidly from the blood. In our studies we have determined the amount which remains in the blood after intravenous injection in rats, and at the same time determined the concentration in the lymph of the thoracic duct. In this way the duration of the purely osmotic effectiveness of the dextrans in the blood is indicated. There is general agreement that the composition of fluid of the

Received for publication June 7, 1951.

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TABLE II. DEXTRAN EXCRETED IN URINE OF RATS

MOLECULAR WEIGHT OF DEXTRAN	MILLIGRAMS EXCRETED AT VARYING TIMES AFTER INJECTION OF 300 MG. OF DEXTRAN	
	6 HOURS	24 HOURS
205,000	25	52
120,000	30	62
85,000	59	110
52,000	72	125
24,000	185	265

tissues is so much like that of fully elaborated lymph as to defy exact definition of boundaries.[11] We have compared the time required for dextrans of different molecular sizes to diffuse out of the blood to the point of extravascular fluid equilibration. We also have determined the influence of molecular size on urinary excretion, the rate of disappearance of dextrans from the blood of the dog, and the rate of urinary excretion. We were unable to detect any conversion of dextrans to glucose in the depancreatized dog.

METHODS

Dextran formed by the action of enzyme-containing filtrates from *Leuconostoc mesenteroides* has a structure similar to starch and cellulose $(C_6H_{10}O)_n$ giving glucose on complete acid hydrolysis. This polysaccharide may be degraded to various sizes by partial hydrolysis. To obtain homogenous fractions of similar molecular weights the dextrans may be precipitated from aqueous solutions by increasing concentrations of ethyl alcohol. By means of ultracentrifugation and measurements of viscosity the fractions of dextrans thus obtained were found to be homogenous with respect to molecular weights.[12] We used five fractions,* the molecular weights of which were determined at the Physico Chemical Institute, Uppsala University, Sweden, to be 205,000, 120,000, 85,000, 52,000, and 24,000. Five milliliters of fresh 6 per cent solutions (300 mg.) of the different fractions were injected by the intravenous route into rats weighing 200 ± 10 Gm. Approximately ninety minutes prior to the collection of blood and lymph, each rat was etherized and a small plastic tube was tied in the thoracic duct.[7] Sufficient lymph (approximately 1 ml.) was collected one hour prior to withdrawal of the specimen of blood by cardiac puncture. Urine was collected from rats by means of an indwelling cystostomy tube for the time desired.

Dextran in the urine was determined by precipitation with copper in alkaline solution as described by Hint and Thorsén.[13] The precipitate is redissolved in acid, and the spectrophotometric density with the anthrone reagent is determined at 620 mμ. We were unable to precipitate dextrans from protein-containing solutions like blood or lymph without marked loss of the dextran. We obtained good determination of dextran added to blood or lymph by direct treatment with the anthrone reagent and correction for the blank (glucose) of the blood or lymph. Determinations of dextran in the blood and lymph were made with centrifuged saline dilutions, 1 to 50 or 1 to 100, by direct treatment with the anthrone reagent and correction for the original glucose content of the blood or lymph. A few determinations were made by the method of Bloom and Willcox[14] which also were satisfactory.

RESULTS

About three minutes were necessary for the intravenous injection of a 6 per cent solution of 5 ml. of dextran into unanesthetized rats as more rapid injections usually caused collapse and often death of the animal. Most rats appeared somewhat depressed for some time after the injection. Specimens of blood removed fifteen, thirty, sixty, and one hundred twenty minutes after

*Made available to us by A. B. Pharmacia, Sweden.

dextran was injected were found to contain from 925 to 640 mg. of dextran for each 100 ml. of blood. Table I shows the amount contained in blood taken three, six, twenty-four or seventy-two hours after the intravenous injection of 300 mg. The concentration in the lymph of the thoracic duct drained during the hour immediately preceding the withdrawal of blood for the same animal is thus the average concentration for this time. Each figure in the table is the average of the results obtained from three or four different animals which did not differ markedly from the figure given.

Table II shows the average amount of dextran found in the urine of unanesthetized rats which received 300 mg. of dextran of the molecular weight indicated.

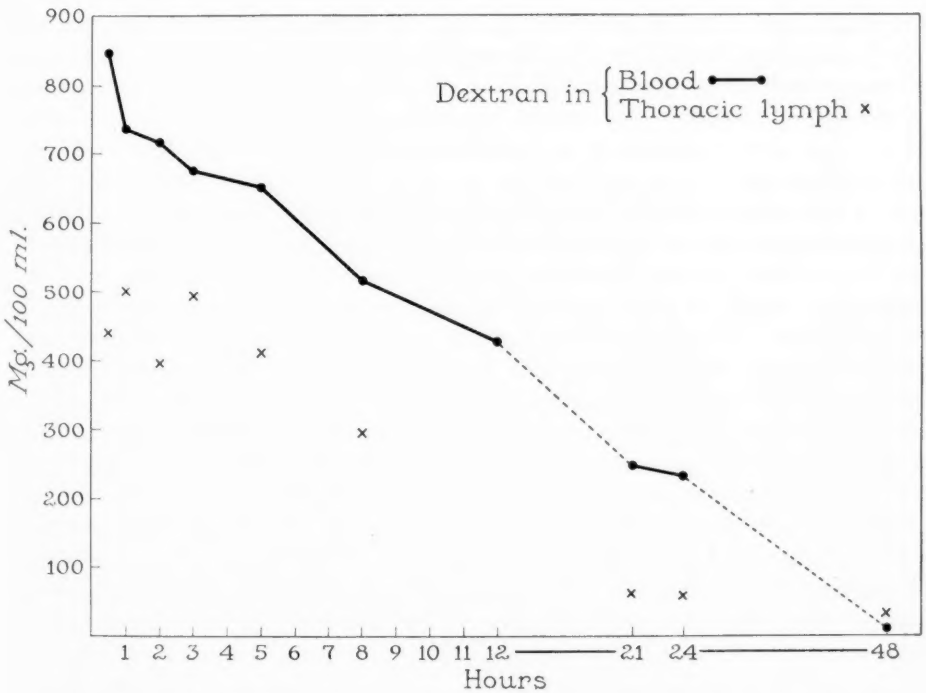

Fig. 1.—Concentration of dextran in blood and lymph of thoracic duct of the dog after intravenous injection of 25 ml. of a 6 per cent solution of dextran Ph for each kilogram of body weight.

Bile collected continuously for twenty-four hours by means of a small plastic tube in the bile duct of four rats which had received 300 mg. of a 6 per cent solution of dextran Ph (of mixed molecular weights) did not contain any dextran. A small amount was found in the intestinal lymph drained six to eight hours after administration of 5 ml. (300 mg.) of mixed dextran by stomach tube.

A dog weighing 8.2 kg. was anesthetized with ether and the thoracic duct was intubated with a small plastic tube so that lymph could be collected. Four hours later when the animal had recovered from the effects of the anes-

thetic 25 ml. of a 6 per cent solution of dextran Ph was injected intravenously for each kilogram of body weight. Specimens of blood were taken at intervals thereafter, and at the time each specimen was taken about 1 ml. of lymph flowed from the thoracic duct fistula in approximately one minute. The amount of dextran contained in the blood and lymph is shown in Fig. 1. The rapid entrance of the substance into the lymph is apparent. In this experiment the animal was allowed to drink fairly large quantities of water to compensate for the fluid lost in the lymph, about 500 ml. each day. The lymph collected therefore was diluted with some water absorbed directly from the gastrointestinal tract, and the figures given for dextran represent minimum values for the lymph.

Two dogs weighing 11 and 9 kg., respectively, were given 417 ml. of a 6 per cent solution of dextran Ph (25 Gm.) intravenously. The urine for the first twenty-four hours after injection contained 15.4 and 16.0 Gm. of dextran. After six days only questionable amounts could be detected in the urine and only a trace remained in the blood. In the six-day period 18.3 and 18.4 Gm. of dextran were recovered in the urine, which represented 74 per cent of the amount injected.

A dog which weighed 11 kg. was depancreatized under ether anesthesia and maintained for several days on insulin and a regular diet. Subsequently, the animal was placed on a fixed regimen receiving exactly 5 units of zinc-protamine insulin at 8:00 A.M. daily and a weighed quantity of mixed diet at the same time. For the next ten days the concentrations of blood sugar were consistent, being approximately 250 mg. per 100 ml. at 8:00 A.M., and approximately 120 mg. per 100 ml. at 4:00 P.M. The urinary glucose was found to be between 14 and 18 Gm. each twenty-four hours. The dog received 25 Gm. of a 6 per cent solution of dextran Ph intravenously and continued on the same regimen of insulin and mixed diet. In the next twenty-four hours the urine contained 34.9 Gm. of carbohydrate of which 18.8 Gm. was dextran and 16.1 Gm. was glucose, which did not indicate a change in the excretion of glucose. On a subsequent date on the same regimen this dog was given 15 Gm. of glucose intravenously; 29.3 Gm. of glucose was excreted in the next twenty-four hours.

COMMENT

From these studies it is apparent that after intravenous administration dextrans rapidly diffuse from the blood. That such rapid diffusion in these experiments was not due to the rapid increase in the volume of plasma attendant on the injection of a relatively large volume of solution was checked in a few additional experiments which showed similar rapid distribution after replacement with solutions of dextran equal in volume to the volume of blood withdrawn. Almost all unanesthetized rats reacted to intravenous injections of dextrans whether or not blood had been previously withdrawn. The rapid diffusion of dextrans from the blood of rats, however, is probably not related to this animal's reaction to dextrans, since the dogs we used had similar rapid diffusion and none of them showed any measureable reaction to injection.

According to Ingelman and Halling[12] the equatorial diameter of dextran molecules is fairly uniform, and the difference in molecular weight is mainly a difference of molecular length. Our data on the urinary excretion of dextrans for both the six and twenty-four hour periods appear consistent with their view. For the sizes of the dextrans tested the excretion was inversely proportional to their molecular weight and not to the square root of their molecular weights, as should be the case were both diameters involved in the molecules of increased weight. The retention of the five different sizes of dextrans in the blood was in the order of 5, 4, 4, 3, and 1, with the largest retention for the largest molecules. Since the same weight of dextrans was administered, the ratio of the number of molecules given was inversely proportional to the molecular weight of the dextrans, and, although more of the larger dextrans was retained in the blood, the osmotic effectiveness of the larger sizes was actually less than that of the smaller sizes. Actually, the dextran of 52,000 molecular weight had the greatest retained osmotic effectiveness because so much more of that of the 24,000 molecular weight was excreted and the larger sizes which were better retained had less total osmotic effect. However, when consideration is given to the number of molecules of the different dextrans retained in the blood in relation to the number injected, our results indicate that the retention in the blood is approximately proportional to the molecular weights of the different fractions.

Considerable doubt is cast on the effectiveness of dextrans (and other macromolecular substances) in holding water in the plasma by their colloidal osmotic effects alone. The fact that the extravascular fluid, as indicated by the concentration of dextran in the lymph, rapidly equals and even exceeds that of the blood largely nullifies any differential in osmotic gradients between the intravascular and extravascular fluids. In our experiments the amount of dextran contained in whole blood was determined, but in no case did dextran contained in the plasma exceed that in whole blood by more than a factor of 1.5. The amount of dextran in the plasma, therefore, exceeded that of the lymph, but the differential was small and could be of little significance in the total osmotic pressure of the plasma. Dextrans and other macromolecular substances, however, are effective in restoring the volume of plasma which has been lost by hemorrhage and in maintaining the volume. Since these substances maintain the volume of plasma for periods which exceed the time of plasma osmotic differential, it would appear that an additional effect is present which could be caused by a secondary alteration in the plasma or an alteration in the extravascular fluid.

Dextrans are excreted in the urine, and almost all of those of small molecular size which are administered may be accounted for. Recovery in the urine of larger sized dextrans, however, is considerably less than those administered. After a time, dextrans are no longer present in the body, and some destruction of them must be postulated as their excretion in the feces appears to be negligible. Dextran is excreted into the gastrointestinal tract and may be reabsorbed from this site. The possibility of its destruction in the intestine remains although incubation of dextran with fecal organisms has not

demonstrated destruction. The possible conversion of dextran to glucose in the body is certainly not rapid. From our experiments it did not seem to be converted to sugar fast enough to alter the concentration of sugar in the blood or urine of the depancreatized dog although the expected response was shown when small amounts of sugar were given.

SUMMARY

Dextrans injected intravenously diffuse from the blood into the lymph and into the urine. In the rat and in the dog the concentration in the lymph from the thoracic duct within a few hours after injection is similar to that of the blood. Dextrans of sizes from 24,000 to 205,000 molecular weight enter the lymph and also appear in the urine. The amount excreted in the urine appears to be inversely proportional to the molecular weight. The rate at which dextran diffuses out of the blood and appears in the lymph also is dependent on molecular size, the larger sizes being retained longer in the blood. On the basis of the total mass of dextrans injected the most effective from the osmotic relationship seems to be the size which is not too rapidly excreted in the urine but which is small enough to exert more osmotic pressure. Dextran does not appear to be converted to glucose in appreciable amounts in the depancreatized dog.

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THE EFFECT OF ENTEROHEPATIC CIRCULATION ON THE BROMSULFALEIN TEST OF HEPATIC FUNCTION

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THE bromsulfalein method of evaluating hepatic function is based on the ability of the normal liver to clear bromsulfalein from the blood stream at a rapid rate. Rosenthal and White[1] recommended injection of 2 mg. of the dye per kilogram of body weight and testing for residual dye in the serum a half hour later. At the Mayo Clinic[2] the amount of dye has been increased to 5 mg. per kilogram, and an hour is allowed to lapse before the serum is tested. Recently, Lorber, Shay, and Siplet[3] have found that an enterohepatic circulation of bromsulfalein exists. They suggested that the absorption of dye from the intestine might interfere with interpretation of the bromsulfalein test, particularly when the larger dose of dye is employed. In an effort to test this possibility the following study was conducted.

METHODS

Twenty-five patients were involved. They consisted of thirteen men and twelve women, ranging from 102 to 230 pounds. Bromsulfalein was introduced directly into the stomachs of twenty patients and into the duodenum of five others. Gastric aspiration had been requested to determine the presence or absence of free hydrochloric acid (it was present in all twenty patients); duodenal drainage was performed in an attempt (unsuccessful) to recover bile from four jaundiced patients and to search for parasites in the fifth.

The dose of bromsulfalein, a commercial solution of 5 per cent strength, was calculated exactly as if it were to be given intravenously; that is, 5 mg. per kilogram of body weight (or 1 ml. of the solution per 10 kg. of body weight). The dye was injected into the gastric or duodenal tube and washed down with about 2 fluid ounces of water. Blood samples were collected at two or more of the following time intervals: fifteen, thirty, forty-five, or sixty minutes.

RESULTS

In none of the twenty-five patients was so much as a trace of dye demonstrated in any specimen of peripheral blood. To determine whether the amount of dye being administered was just below the level required for it to appear in the blood, two additional patients received, by gastric tube, twice the dose previously employed (10 mg. per kilogram of body weight); again no dye was evident in the serum during the one hour test period.

CONCLUSIONS

The enterohepatic circulation of bromsulfalein as reported to exist by Lorber, Shay, and Siplet[3] would not seem to be a cause for concern in evaluation of the clinical bromsulfalein test for hepatic function.

From the Division of Clinical Laboratories, Mayo Clinic.
Received for publication, June 8, 1951.

We wish to acknowledge the technical assistance of Miss Agnes Hand, Mrs. Helen Woodruff, and Mrs. Florence Grue.

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VENOUS CATHETERIZATION FOR CONTINUOUS PARENTERAL FLUID THERAPY

Use of Heparin in Delaying Thrombophlebitis

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IN A preceding paper[1] a method of venous catheterization was described, and it was observed that continuous administration of parenteral fluids into the superficial veins through plastic catheters may be expected to be limited to an average duration of three days due to development of thrombophlebitis.

Recently efforts to attain more extended, uninterrupted fluid administration have included catheterization of the innominate and iliac veins.[2]

By incorporating heparin into intravenous fluids, a relatively high concentration of heparin can be created at the site of venipuncture, while resulting in a relatively low systemic effect. Such heparin administration is the object of the present study, in an attempt to prolong intravenous infusions by preventing thrombophlebitis.

EXPERIMENTAL

Plastic venous catheters were employed to administer intravenous fluids continuously to medical and surgical patients. Conditions were similar to those of the previous series,[1] with the exception that 10 mg. of heparin* was added to each liter of fluid. Five per cent glucose, physiologic saline, or blood was infused into the superficial veins of the extremities at an average rate of 3 liters per day. The patients were allowed to move the extremities freely. Venoclysis through each catheter was maintained until thrombophlebitis developed or until the patient's requirement for parenteral fluid was terminated.

Because of the possibility of increased irritation due to protein hydrolysates and 10 per cent glucose, patients receiving large amounts of these solutions were not included in the series.

Small concentrations of heparin activity in the circulating blood can be estimated quantitatively by the heparin-protamine titration method.[3] In order to determine whether systemic accumulation of heparin effect occurred in this series, heparin-protamine titrations were performed on patients at the beginning of and at intervals during the period of observation. Determinations were made at two-day intervals on fifteen patients and at three-day intervals on five patients. In addition thirty-five blood-clotting times were determined by the Lee and White method on ten patients, before and during fluid administration.

RESULTS

In Table I a series of twenty-six patients receiving fluid containing heparin is compared with the previously reported series of sixty-two patients[1] who received no heparin. The longest infusion without heparin was continued for seven days, whereas in the present series two patients were maintained for as

From the Third Medical Service Laboratory, St. Luke's Hospital.
Aided by grants from Baxter Laboratories, Inc., Morton Grove, Ill., and Commercial Solvents Corp., New York, N. Y.
Received for publication, June 11, 1951.
*130 international units per milligram.

long as twenty days. The incidences of thrombophlebitis observed on consecutive days were arranged into cumulative percentages. The observed percentages were treated according to the methods of Dragstedt and Lang[4] and Bliss,[5] and the standard errors were estimated by the method of Miller and Tainter.[6] It was ascertained that 50 per cent of patients so treated with heparin may be expected to have developed thrombophlebitis at 8.6 days, as opposed to 3.3 days for the non-heparin treated group. Statistical analysis revealed a significant difference in the time of development of thrombophlebitis in the two groups of patients.

TABLE I. CUMULATIVE INCIDENCE OF THROMBOPHLEBITIS IN PATIENTS RECEIVING CONTINUOUS INTRAVENOUS FLUID ADMINISTRATION THROUGH PLASTIC CATHETERS, WITH AND WITHOUT THE ADDITION OF HEPARIN

DURATION OF FLUID ADMINISTRATION IN DAYS	HEPARIN ADDED, 10 MG./L.			WITHOUT HEPARIN		
	NO. OF PATIENTS*	THROMBOPHLEBITIS		NO. OF PATIENTS*	THROMBOPHLEBITIS	
		NO.	PER CENT		NO.	PER CENT
1	26	0	0	62	1	1.6
2	26	0	0	42	3	7.2
3	26	1	3.8	28	12	42.8
4	25	4	16.0	26	19	73.0
5	25	6	20.0	26	22	84.6
6	20	6	30.0	25	22	88.0
7	17	7	41.2	23	23	100
8	15	7	46.7			
9	13	8	61.5			
10	13	8	61.5			
11	12	8	66.7			
12	12	9	75.0			
13	12	10	83.3			
14	12	10	83.3			
15	12	10	83.3			
16	12	10	83.3			
17	12	10	83.3			
18	12	10	83.3			
19	12	10	83.3			
20	12	11	91.5			

	Heparin added, 10 mg./l.	Without Heparin
Median duration of venoclysis sufficient to produce thrombophlebitis	8.6 days	3.3 days
Standard error	0.42 day	0.14 day

*Each figure includes all patients receiving fluid for the designated number of days plus the total number which had developed thrombophlebitis prior to that day, i.e., in the heparin treated series, eight patients developed thrombophlebitis prior to the twelfth day and four received fluid for twelve days.

Heparin-protamine titrations demonstrated no heparin activity in the blood of sixteen patients. In two patients 1.7 micrograms per milliliter* was indicated at one interval (three days and eleven days, respectively). Two others, in whom concentrations of 3.3 micrograms per milliliter were obtained before treament, showed no increase in this level during heparin administration. In the four patients showing abnormal heparin titration values, no evidence of bleeding was observed.

*1.7 micrograms per milliliter represent the mean blood level obtained by heparin-protamine titration in 70 kg. patients fifteen minutes after a single intravenous heparin injection of 10 milligrams.

The Lee-White clotting times were without significant variation from the patients' control values.

Plugging or kinking of the catheters occurred rarely.

SUMMARY

Patients were studied who received intravenous fluids continuously into a superficial vein through a plastic venous catheter. Development of thrombophlebitis was significantly delayed when ten milligrams of heparin was incorporated into each liter of fluid. The average patient thus treated may be expected to develop thrombophlebitis as a consequence of 8.6 days of continuous fluid administration, as compared with 3.3 days for patients not receiving heparin. No significant alteration was detected in the heparin-protamine titration test or the Lee-White clotting time due to this heparin dosage.

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ESTIMATION OF CARDIAC OUTPUT AND EVANS BLUE SPACE IN MAN, USING AN OXIMETER*

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THE validity of the dye-dilution method for measuring cardiac output[1, 2] has been confirmed,[3, 4] and the same principle has been widely used for the estimation of blood volume.[5] The suitability of Evans blue dye as the indicator for such determinations has much experimental support.[6-9] The dye-dilution and direct Fick procedures are the two most generally accepted methods providing absolute determinations of cardiac output in the intact human being. The dye method has not, however, been widely applied because of the time-consuming nature and technical difficulty of the Hamilton procedure. Such problems would be minimized if a method were available whereby the concentration of the dye in arterial blood could be measured continuously . This paper presents such a method, utilizing a cuvette oximeter for whole blood.[10-13]

MATERIALS AND METHODS USED

The Stewart-Hamilton method for measuring cardiac output may be outlined as follows: The dye is injected intravenously, arterial blood samples are collected at one-second intervals for about one minute thereafter, the concentration of dye in the plasma of each sample is measured, and the logarithm of the concentration of each sample is plotted against the elapsed time after injection of the dye. The points so plotted form a curve, with an initial ascent as the dye appears in increasing concentration, followed by a downward deflection. The first portion of the descending limb approximates a straight line before it is again deflected upward by recirculation of the dye. When this straight line is extrapolated to the base line (0 concentration), a measurement of the time required for the initial passage of the dye through the heart and lungs is obtained and the average concentration of dye over this period of time may be derived. Therefore, if the amount of dye injected is known, the rate of flow (systemic output of blood from the heart) can be calculated from the relationship:

$$F = \frac{60\ I}{ct}$$

where F = flow in liters per minute, I = milligrams of dye injected, and c = the average concentration of dye in milligrams per liter existing over t seconds, the time of initial passage of dye. Evidence has been presented to show that the flow of water through a system of tubes and bulbs can be calculated by the dye-injection procedure.[14] Mathematical analyses of the formula for expressing time-concentration curves of indicators in circulating arterial blood have recently been carried out by Nylin and Celander[15] and by Cyvin.[16]. The error of the method is held to be approximately the same as that of the Fick determination, or 11 per cent.[4]

During the course of studies undertaken to make an objective measurement of circulation time by injecting methylene blue or Evans blue intravenously and recording its appearance in arterial blood by use of a selenium barrier-layer photocell oximeter,[17] it was observed that

Received for publication, June 11, 1951.

*This report contains data from a thesis submitted by Dr. Nicholson to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

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the changing concentration of dye in whole arterial blood, as recorded photokymographically, produced a continuous curve similar in configuration to that obtained by connecting the separate points representing the concentration of dye in plasma, plotted semilogarithmically according to the method outlined in the preceding paragraph. The ease with which such a record could be obtained suggested investigation of the procedure as a possible method for determining cardiac output. Further, evidence of equilibration of the dye in arterial blood within 90 seconds after intravenous injection (in normal subjects) was furnished by the early disappearance of fluctuations in the transmission of light through the circulating blood-dye mixture, with stabilization at a new and reasonably constant level of light transmission that was a little lower than that of the blood before dye was added. This observation suggested the added possibility of estimating Evans blue space, with the attractive features of being able to make the measurement in whole blood and with a minimal lapse of time between injection and measurement, thus avoiding hematocrit determinations, the use of a factor to account for plasma trapped between the centrifuged erythrocytes, and extrapolation to zero time of a slope representing an average rate of disappearance of dye from the blood stream.

The principle on which these studies were based is that Evans blue dye absorbs light maximally at a wavelength of 620-630 mμ, which lies in the "red" range, and absorbs very little light at 800 mμ, which is in the "infra-red" range. The oximeter used had two photocell-filter assemblies, one having a peak response in the red range (640 mμ) and the other a peak response in the infra-red range (800 mμ).[18] This instrument was designed primarily to measure the oxygen saturation of whole blood, the red photocell being sensitive to changes in the oxygen saturation of hemoglobin and the infra-red photocell being sensitive to changes in the total amount of hemoglobin regardless of saturation; it is readily seen, however, that the red photocell would also be sensitive to changes in the concentration of Evans blue dye. The instrument used in these studies was the cuvette oximeter for whole blood previously described by Wood,[10] connected in a circuit which permitted simultaneous single and double-scale operation of the instrument.[11] With the infra-red photocell monitoring the constancy of hemoglobin concentration present, and with the subject breathing 100 per cent oxygen to minimize fluctuations in the oxygen saturation of arterial blood, it appeared likely that changes in light absorption produced by the addition of Evans blue dye to such blood might be measurable in terms of concentration of the dye. The data to be presented include a demonstration of the degree of variability encountered in making these measurements.

A more detailed description and illustrations of the materials used appear elsewhere.[19] A 2 per cent aqueous solution of Evans blue dye (Eastman Kodak Company) was used in all cases, the amount injected ranging from 30 to 60 mg. (1.5 to 3 c.c.). The most commonly used dose was 40 milligrams. No untoward effects were seen in more than 150 injections, except an occasional mild bluish discoloration of the subject when the dose exceeded 40 milligrams. The syringe used for the injection was held in a special clamp permitting accurate delivery of the desired amount of dye, and was connected by vinylite tubing to a special three-way adapter to which a 19-gauge intravenous needle was attached. A syringe containing approximately 5 c.c. of sterile heparinized saline solution, used for flushing the dye into the vein, was attached by vinylite tubing to the third limb of the adapter.

The arterial sampling system is illustrated in Fig. 1. The arterial needle was 20-gauge and internally polished. It was inserted into the radial artery with the subject under procaine analgesia, and sampling of blood was carried out using a technique similar to that described by Wood and associates.[20] Changes in light transmission occurring in the cuvette oximeter were transmitted to d'Arsonval-type galvanometers without electronic amplification, and recorded continuously on 18-inch photosensitive paper moving at 5 mm. per second utilizing an oscillographic recording assembly described previously.[11]

I. *The Dye-Injection Procedure.*—This was carried out as follows: With the subject supine, an indwelling needle was placed in the right radial artery and connected with the arterial sampling system[12] (Fig. 1). Venipuncture was performed using a suitable vein in the antecubital space of the left arm; the needle was taped in place and connected with the dye-injection assembly.

Sterile heparinized saline solution under pressure was used for flushing the arterial sampling system. All subjects breathed 100 per cent oxygen throughout the procedure, using a closed system incorporating a recording spirometer and soda lime for carbon dioxide absorption. Respiratory rate was recorded as variations in pressure using a strain gauge manometer[21] connected with the oral airway. Intra-arterial pressure was recorded by a similar instrument in the arterial sampling system.[12] Electrodes for recording the electro-

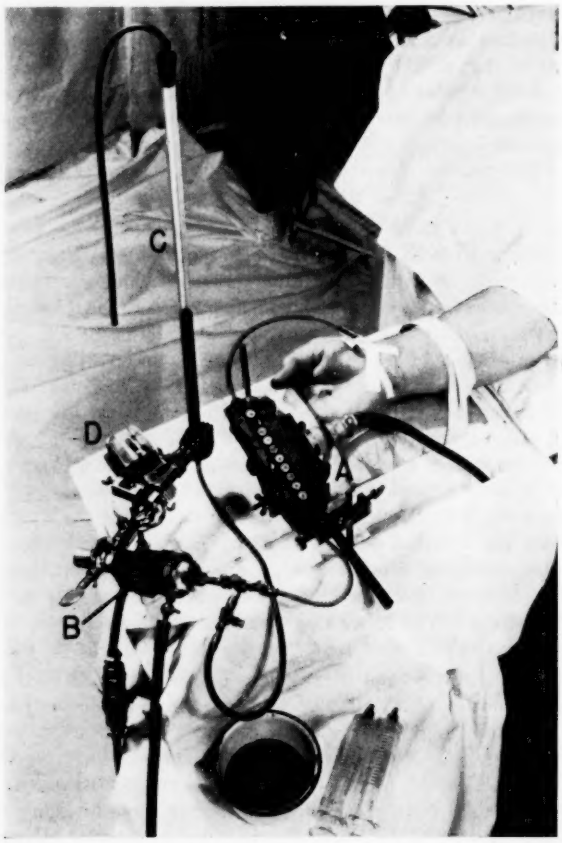

Fig. 1.—Arterial sampling system. Needle in radial artery is connected by a glass adapter and polythene tubing to the cuvette oximeter, *A*. Intra-arterial pressures are recorded from the strain gauge manometer, *B*. Blood flows through the oximeter and through a side-arm connection into buret, *C*, to the top of which, using a syringe, suction may be applied as needed for an even rate of flow. Each cubic centimeter of flow into the buret is recorded by an observer using the signal switch, *D*. Electrical connections from the cuvette oximeter, strain gauge manometer and signal magnet are made through a junction box and conduit to an oscillographic recording assembly[11] located in an adjacent room.

cardiogram and heart rate were taped to the subject's thorax. Connections from the various instruments were made through a junction box to galvanometers in the adjacent recording room.[11] After the subject had been breathing 100 per cent oxygen for at least five minutes, a 30 c.c. sample of arterial blood was drawn into a siliconed, lightly-oiled syringe containing a small amount of powdered heparin. This blood was saved for the preparation of known dilutions of dye in whole oxygenated blood to allow quantitative calibration of the deflections produced by known concentrations of the dye in blood as recorded by the cuvette oximeter.

Blood was now allowed to flow from the radial artery through the cuvette oximeter and into a graduated, 25 c.c. buret, each cubic centimeter of flow being marked on the record

with a signal magnet. Suction was applied as desired to the top of the buret to insure an even rate of flow. After several cubic centimeters of flow, the dye was injected intravenously as rapidly as possible and flushed in with saline solution. Arterial sampling was continued at an even pace for approximately ninety seconds (longer in patients with heart failure, in whom equilibration of dye in blood is slower). When necessary, flow was allowed to continue beyond the 25 ml. mark, the blood in this event being diverted through a side-arm connection into a discard basin. A determination of the subject's rate of oxygen consumption was made during the period of flow of arterial blood. Approximately 5 minutes after the injection of dye, a second period of arterial blood flow was allowed, lasting for thirty seconds. Flow was then stopped, administration of oxygen was discontinued, and the procedure was terminated.

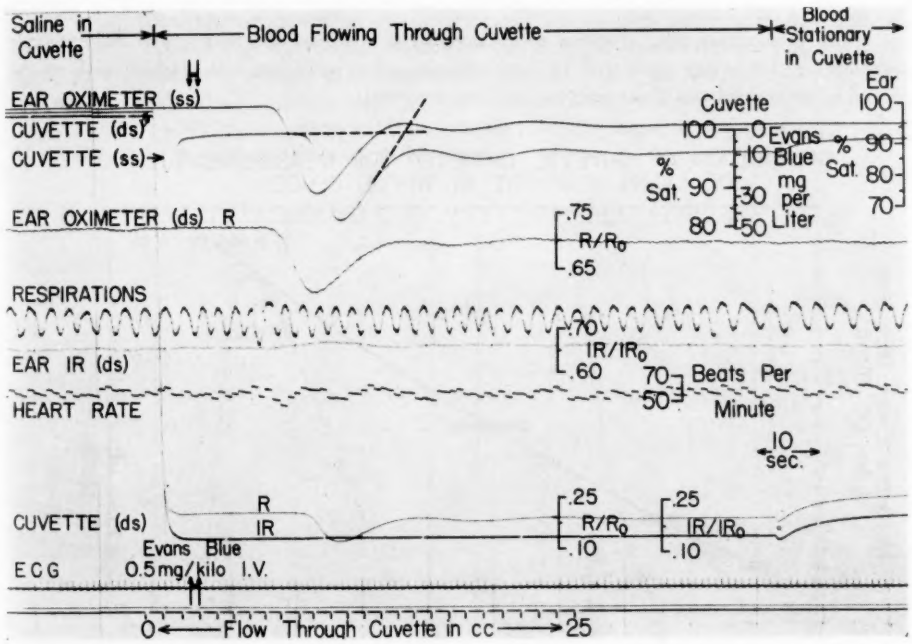

Fig. 2.—Continuous recording of dye concentration in arterial blood and other physiologic variables after nearly instantaneous injection of 40 mg. of Evans blue dye into left antecubital vein of a normal subject. Dye concentration was recorded by means of ear oximeter attached to left ear and cuvette oximeter attached to an indwelling needle in the radial artery. The subject was breathing 100 per cent oxygen, so that constant and complete saturation of the arterial blood with oxygen was assured. Simultaneous single-scale and double-scale operation of the cuvette and earpiece were used. Galvanometer traces labeled ss (single-scale) are recordings of the difference in output of the red-sensitive and infra-red-sensitive photocells of ear and cuvette oximeters, respectively. Galvanometer traces labeled ds (double-scale) are individual recordings of the output of the red-sensitive and infra-red-sensitive photocells of earpiece and cuvette. R/Ro indicates the ratio of red light transmitted through the blood-containing ear or blood-filled cuvette to the light transmitted through the bloodless (pressurized) ear or saline-filled cuvette. IR/IRo is the similar ratio for near infra-red light transmission through the ear or cuvette. The logarithms of the reciprocals of these ratios are related to the optical density of the transilluminated blood contained in the ear and the cuvette. The significance of the broken lines on the "cuvette (ss)" curve is discussed in the text.

II. *Procedure for the Oximetric Calibration of Dye in Blood.*—Standard aqueous dye solutions were prepared for calibration purposes. These contained 125, 62.5, 31.3, 15.6 and 7.8 milligrams of Evans blue dye per liter of water, respectively. Five aliquot samples of the arterial blood, each of 5 ml., drawn just before the dye-injection procedure, were pipetted into 50 ml. test tubes, the blood sample being frequently agitated to prevent settling of erythrocytes. Using a 1 ml. tuberculin syringe held in a clamp which permitted accurate withdrawal and expulsion of 0.2 ml. of fluid, 0.2 ml. of isotonic saline solution was added to the

5 ml. "control" blood sample. Syringe and needle were then flushed with the most dilute of the standard aqueous dye solutions, and 0.2 ml. of this solution was drawn up and added to the next 5 ml. blood sample. This gave an approximate concentration of 3.131 milligrams Evans blue dye per liter of whole blood; the exact concentration was obtained by correcting this figure for the true volume of the blood-dye mixture (5.2 ml. instead of 5 ml.) and for the actual amount of dye solution delivered from the syringe with the clamp set at 0.2 ml. delivery, this amount having been determined gravimetrically. The blood-dye solutions made up in this manner, containing 25, 12.5, 6.25, and 3.13 milligrams Evans blue dye per liter of whole blood, respectively, embraced the range of dye concentrations ordinarily encountered in the arterial blood of subjects after intravenous injection of 20 to 50 mg. of the dye.

The control blood sample and the four known dilutions were then drawn up into clean, oiled syringes and run through the cuvette oximeter in succession at a slow, uniform rate of flow while continuous photographic recordings were made of the single and double-scale galvanometer deflections produced by each sample. The oximeter was flushed with saline solution after each of the blood samples had been recorded.

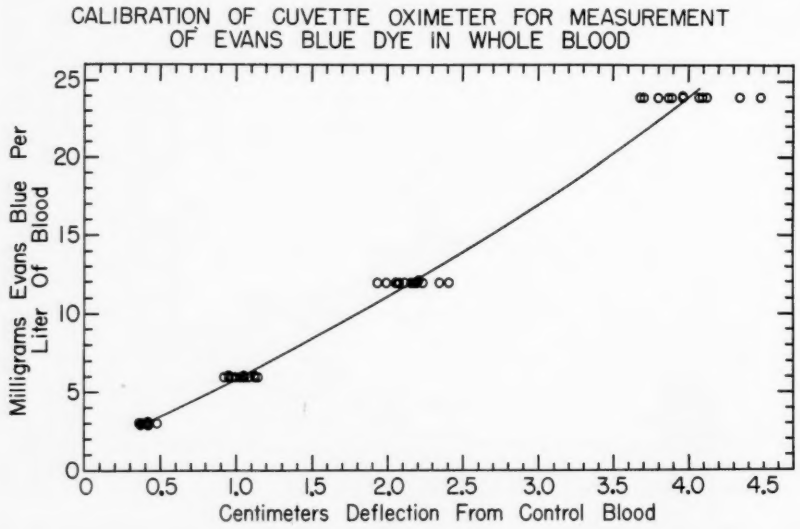

Fig. 3.—Calibration of cuvette oximeter for measurement of Evans blue dye in whole blood.

III. *Procedure for Measurements and Calculations.*—The single-scale galvanometer deflections produced by the known blood-dye dilutions were measured from the photographic record, using the control-blood deflection as the base line (Fig. 2). For uniformity, each measurement of such a deflection on a given record was made at a point representing a lapse of time from entry of the blood into the cuvette that was constant from sample to sample; the time interval used was commonly twenty seconds. A calibration curve was plotted using milligrams of dye per liter of whole blood as the ordinate and centimeters of deflection from the control-blood level as the abscissa (Fig. 3).

The following sequence was usually seen in the continuously recorded dye curves obtained in the study of normal subjects (Fig. 2): (1) a period of time, ranging from ten to twenty seconds, between injection of the dye and the beginning of an alteration in light transmission through arterial blood flowing past the oximeter; (2) a smooth and fairly rapid downward deflection of the "single scale" and "red" galvanometer traces, signifying an increase in concentration of the dye, followed by: (3) a smooth and slightly slower upward deflection

or decrease in dye concentration as the dye was partially cleared from the heart and lungs on its first circuit, and (4) a small secondary downward deflection representing systemic recirculation of the dye. Stabilization of light transmission at a new level, slightly lower than that of undyed blood, was seen soon thereafter.

Measurement of the dye curve was made according to the following steps:

1. A straight, horizontal line was projected from the level of light transmission through undyed blood, of sufficient length to cover the duration of the primary dye curve. This was the "zero" line from which subsequent measurements were made.

2. The primary dye curve was marked off at one-second intervals to a point where beginning recirculation was evident, and the dye concentration in arterial blood at each second was determined, utilizing the subject's calibration curve for dye in blood. The logarithms of these values for concentration were plotted against time in seconds. When so plotted, the points along the disappearance slope of the dye curve were seen to fall in a straight line until recirculation began (Fig. 4). Extrapolation of this straight line to the time base was then accomplished after the method of Hamilton and others. The time interval (t) between the appearance of the dye in arterial blood and the intersection of the extrapolated line with the time base (0 concentration) supposedly represents the time required for the initial passage of the injected dye through the heart and lungs.

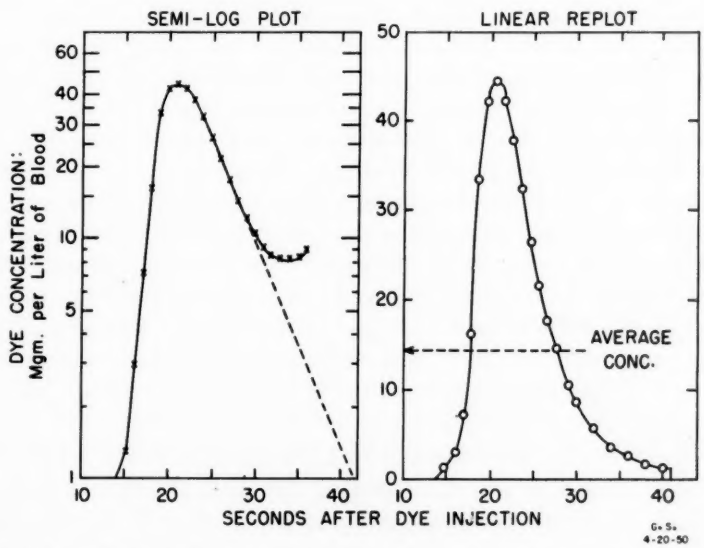

Fig. 4.—Semilogarithmic plot and linear replot of the dye curve shown in Fig. 2.

3. A linear replot of the extrapolated dye curve was made (Fig. 4), and the average concentration of dye during this primary dye curve was determined by planimetry. The value so obtained supposedly represented the average concentration of dye in the volume of blood pumped through the heart and lungs during the period of the initial passage of the dye. Thus, knowing the volume of blood and the time required for its passage through the heart and lungs, one could calculate the rate of blood flow (cardiac output) through this portion of the circulation.

After stabilization of the blood-dye level had occurred (about ninety seconds after the injection, Fig. 2), the concentration of the dye in arterial blood was measured. The Evans blue space was then calculated by dividing this value into the number of milligrams of dye injected. In determining the stabilized concentration of dye in blood a small correction was applied for the slight electrical drift observed in the cuvette oximeter under conditions of prolonged flow of blood through this instrument.

RESULTS

I. *Calibration of the Cuvette Oximeter in Terms of Milligrams of Evans Blue Dye per Liter of Whole Oxygenated Blood.*—Photoelectric determinations were carried out on two series of known dilutions of Evans blue dye in blood. All determinations at a given level of dye concentration were made on samples of blood from different individuals. These samples covered a range of hemoglobin concentrations extending from 4.1 Gm. per 100 ml. (28 per cent) to 24.2 Gm. per 100 ml. (162 per cent), the extremes representing samples of blood that were artificially diluted and concentrated, by removing or adding plasma, respectively. All samples were fully saturated with oxygen, artificially in the case of venous blood samples or, in the case of arterial blood samples, by having the subject breathe 100 per cent oxygen for at least five minutes before the sample was drawn. The results are shown in Table I and Fig. 3. The difference in deflections produced by the same blood-dye concentrations in the two series was apparently due to changing the polythene tubing in the cuvette oximeter between series 1 and series 2. The relationship of the concentration of dye in whole blood to centimeters of galvanometric deflection approached linearity in the lower ranges of concentration encountered in most measurements in vivo. The standard deviation of individual photoelectric determinations from the mean value for the series ranged from 4 to 11 per cent at the various levels of concentration used (Table I). The quantitative measurement of Evans blue dye in whole blood with an oximeter requires constancy of the oxygen saturation of blood, since both reduced hemoglobin and Evans blue dye absorb light in the red range, thereby presenting the likelihood that changes in the oxygen saturation of blood could be erroneously interpreted as being changes in the concentration of dye. When the same known dilutions of dye as those appearing in Fig. 3 were made up in blood not fully saturated with oxygen and compared with control readings on an undyed sample of the same blood, the points so derived fell to the left of the range shown, their departure from this range increasing with greater desaturation of the blood.

Not apparent from Fig. 3 is the fact that dilutions of dye in a given subject's blood tended to parallel the mean curve rather than to show a random

TABLE I. VARIABILITY OF PHOTOELECTRIC DETERMINATIONS OF THE CONCENTRATION OF EVANS BLUE DYE IN FULLY OXYGENATED WHOLE BLOOD, USING THE CUVETTE OXIMETER

	CONCENTRATION OF DYE IN STANDARD DILUTIONS, MG. PER LITER OF BLOOD			
	3.13	6.25	12.5	25
Series 1 No. of different specimens of blood	8	10	11	11
Standard deviation, per cent	±10.9	±4.6	±3.6	±3.7
Series 2 No. of different specimens of blood	10	13	15	12
Standard deviation, per cent	±5.4	±5.8	±6.0	±7.0

scatter within the range shown. Because of this fact, and of the known error of the average curve, all calculations of cardiac output and Evans blue space were made using data based on the subject's individual calibration curve rather than from the average curve.

II. *Accuracy of the Dye-Dilution Method in Estimating Blood Volume in Vitro.*—In order to learn the degree of accuracy with which a calculation of blood volume could be made in vitro, using an oximeter to estimate the degree of dilution of the dye in whole blood, fifteen measured volumes called "unknowns" were prepared from the oxygenated blood of four different individuals, and a known amount of dye was added to each, sufficient to produce a concentration in the range encountered during determinations in vivo. The concentration of dye in a blood sample from each "unknown" volume was measured using the cuvette oximeter, and a calibration curve was determined

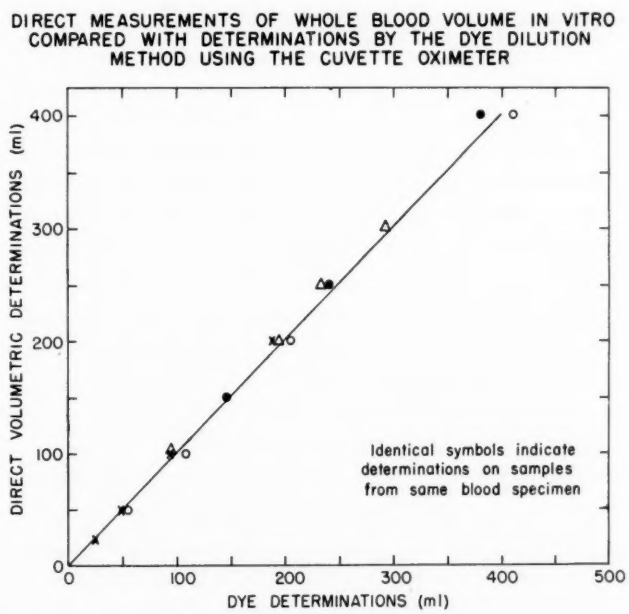
DIRECT MEASUREMENTS OF WHOLE BLOOD VOLUME IN VITRO COMPARED WITH DETERMINATIONS BY THE DYE DILUTION METHOD USING THE CUVETTE OXIMETER
DIRECT VOLUMETRIC DETERMINATIONS (ml)
DYE DETERMINATIONS (ml)
0
100
200
300
400
500
Identical symbols indicate determinations on samples from same blood specimen

Fig. 5.—Direct measurements of whole blood volume in vitro compared with determinations by the dye dilution method using the cuvette oximeter.

for each of the four blood specimens as outlined. Calculation of volume was made by dividing the amount of dye originally added to the "unknown" by the concentration of dye measured in the blood sample, and the results were compared with the directly measured volume of the "unknown." The standard deviation of the differences between photoelectric and volumetric determinations was 5 per cent with no apparent systematic variation (Fig. 5).

III. *Determination of Cardiac Output.*—Cardiac output determinations were made by the method previously outlined in eleven normal young adult subjects. Of these, ten fulfilled the arbitrary criteria set by others[22, 23] to define a normal, near-basal state, that is, a metabolic rate less than +10 per cent

and a heart rate less than 82 beats per minute. The average cardiac index* of the ten normal, near-basal subjects was 3.2 liters per minute per square meter with a range of 2.0 to 4.3, a standard deviation of 0.6 and a standard error of the mean of ±0.2 liter per minute per square meter. A comparison of this series with two others by different investigators[22, 23] who employed the direct Fick method, using the technique of right heart catheterization, is shown in Table II.

TABLE II. THE CARDIAC INDEX IN NORMAL ADULT HUMAN SUBJECTS*

	STEAD AND ASSOCIATES[23] 1945	COURNAND AND ASSOCIATES[22] 1945	CURRENT SERIES 1950
Number of subjects	11†	13	10
Method used	Direct Fick	Direct Fick	Dye
Cardiac index (l./min./sq.m.)	3.18	3.12	3.17
Range	2.4-3.9	2.12-4.01	1.96-4.32
Standard deviation	0.55	0.48	0.64
Standard error of mean	±0.17	±0.13	±0.20

*Heart rate less than 82 beats per minute, metabolic rate less than +10 per cent.
†These normal subjects were part of a larger reported series which included hospital patients.

In addition, both dye and direct Fick determinations of cardiac output were done in a small series of patients with cardiovascular abnormalities. The measurements were made simultaneously in four, and at different times (but under similar conditions) in four others. The results, in terms of cardiac index, are shown in Fig. 6. The dye value varied from +23 per cent to −17 per cent of the Fick value for cardiac index without apparent systematic difference. This range is within the reported limits of the combined errors of the two methods.[3] It should be noted that these dye determinations were made on abnormal curves. The extrapolation of a dye-disappearance slope was carried out empirically, regardless of the time relationships or the lack of evidence for systemic recirculation of the dye. Use of this type of extrapolation on "abnormal" curves has not been justified mathematically.

An attempt was made, on an empirical basis, further to simplify the estimation of cardiac output by making all measurements directly on the photographic record, thus eliminating the ordinarily required steps of constructing a semilogarithmic plot of the time-concentration data derived from the photographed curve. A "best visual line" was arbitrarily extrapolated, on the photographic record, from the straightest segment of the disappearance slope of the primary dye curve, to the point of intersection with a horizontal line projected from the control-blood level (broken lines in Fig. 2). The mean concentration and duration of this "primary" dye curve were thus obtained directly from the photographic record. Calculation of output was made using the formula previously stated. A comparison of the values for cardiac index so derived with the values obtained by measuring according to the Hamilton method appears in Table III. The values for cardiac index obtained from direct measurements on the photographic records averaged 124.4 per cent of

*Cardiac index: Cardiac output in liters/min./body surface area in square meters.

the values obtained by replotting concentration against time on a semilogarithmic scale, with a range of 118 per cent to 130 per cent and a standard error of the mean of ±1.4 per cent.

Dye curves recorded from seven patients with cardiac abnormalities were treated in the same manner. A comparison of results is shown in Table IV. The photographic values averaged 127 per cent of the replot values, with a somewhat greater degree of variability than in normals. The convenience of making all measurements directly on the photographic record suggests the practical possibility of using this method and correcting the result by the average difference between this and the usual procedure, recognizing the result as an approximation.

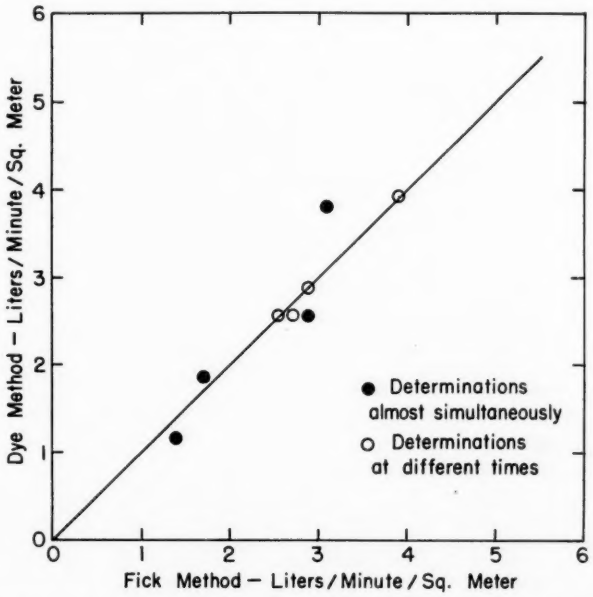

Fig. 6.—Comparison of cardiac index values determined by the direct Fick method and by the dye dilution method in the same patients.

IV. *Determination of Evans Blue Space.*—In twelve normal subjects a measurement of Evans blue space was made at a point on the record averaging ninety-one seconds after injection of the dye, and in eleven of these subjects a second measurement was made at points ranging from five to eight minutes after the injection. The average Evans blue space measured at an average of 91 seconds after injection of the dye was 65 ml. per kilogram and at five to eight minutes it was 72 ml. per kilogram (Table V). The second determination in every case gave a larger value than the first. The average difference of 6 ml. per kilogram between the determinations carried out at varying intervals after injection was statistically significant (P value <0.001). The average 91-second and five- to eight-minute values are near the lower limits of the range commonly considered as normal for blood volume (60 to 90 ml. per kilogram). In three patients the 91-second determination of Evans blue

TABLE III. CARDIAC INDEXES OF 10 NORMAL SUBJECTS* CALCULATED (A) FROM THE CONTINUOUSLY RECORDED DYE CURVES, AND (B) BY REPLOTTING THE SAME CURVES WITH THE DYE CONCENTRATION ON A LOGARITHMIC SCALE

SUBJECT	CARDIAC INDEX 1./MIN./SQ.M.		PER CENT RATIO (100A)
	PHOTO (A)	REPLOT (B)	B
1	2.53	1.96	129
2	3.50	2.77	126
3	3.53	2.98	118
4	3.76	2.89	130
5	3.81	3.08	124
6	3.95	3.25	122
7	3.97	3.06	130
8	4.46	3.75	119
9	4.50	3.63	124
10	5.29	4.32	122
Mean	3.93	3.17	124.4
Standard error of mean	±0.23	±0.20	±1.36

*Only subjects who were in a relatively basal state were included (heart rate <82 beats per min.; metabolic rate <+10 per cent).

TABLE IV. CARDIAC INDEXES OF PATIENTS CALCULATED (A) FROM THE CONTINUOUSLY RECORDED DYE CURVES, AND (B) BY REPLOTTING THE SAME CURVES WITH THE DYE CONCENTRATION ON A LOGARITHMIC SCALE

PATIENT	ABNORMALITY	CARDIAC INDEX 1./MIN./SQ. M.		PER CENT RATIO (100A/B)
		PHOTO (A)	REPLOT (B)	
A	Mitral stenosis, chronic heart failure	1.20	0.89	135
B	Patent ductus arteriosus	1.66	1.16	143
C	Shunt (right to left)	2.92	2.57	114
D	Patent ductus arteriosus	3.31	2.57	129
E	Interatrial septal defect	2.97	2.58	115
F	Tetralogy of Fallot	4.75	3.80	125
G	Normal heart, nonbasal state	7.20	5.59	129
Mean				127
Standard deviation				10.4
Standard error of mean				±3.9

space amounted to 76, 85, and 88 per cent, respectively, of blood volume determinations done one or two days previously by a standardized Congo red method[24] in the same individuals. The five- to eight-minute determinations were 80, 92, and 94 per cent, respectively, of the Congo red values for blood volume. (The Congo red method in question does not incorporate extrapolation of a dye-disappearance slope in the calculation of blood volume, the concentration of dye in the plasma of a single sample of blood drawn five minutes after injection of the dye being used as a measure of the dilution that would have existed supposing the dye to have been uniformly distributed in the circulating blood at the moment of injection.)

ADVANTAGES OF METHOD

Salient points in favor of the method are the continuous recording technique, eliminating the need for measuring dye in the plasma of many consecutive small samples of blood, and the fact that determinations of the concentration of dye are made in whole blood rather than in plasma. The con-

TABLE V. EVANS BLUE* SPACE IN NORMAL SUBJECTS: VARIATION WITH TIME AFTER DYE INJECTION

		12 SUBJECTS	11 SUBJECTS	DIFFERENCE
Time of taking succesive samples after dye injection, seconds	Average	90.8	357	--
	Range	70-100	320-480	--
	Standard error of mean	±2.3	±15.0	--
Evans blue space, ml./kg.	Average	65.4	72	6.1
	Range	53-82	57-89	2-14
	Standard error of mean	±2.4	±3.0	±1.2

*Measured in whole arterial blood with a cuvette oximeter.

tinuous recording of changes in the concentration of dye furnishes an accurate picture of the contour of the primary dye curve, giving the method an added practical usefulness in contributing information of diagnostic value in patients with certain types of cardiovascular disorders.[19] In addition, simultaneous recordings of dye curves were made with the cuvette oximeter and with a single-scale ear oximeter.[10] The close similarity in contour of the tracings obtained by the two instruments suggested a further simplification of the method that might eliminate the necessity for arterial puncture.[25] In the case of both the cuvette and ear oximeters an objective measurement of arm-to-ear or arm-to-arm appearance times, mean circulation times, and, if an evident recirculation curve is present, a measure of mean systemic recirculation time is provided.[19] The mean circulation time can be used in conjunction with the cardiac output to calculate a value supposedly related to the volume of blood contained in the central portion of the circulation (chiefly the heart and lungs).[26]

For the measurement of Evans blue space, the method, though not directly comparable with other dye procedures widely used to measure blood volume, appears to offer fewer sources of error than do dye procedures incorporating the measurement of dye in plasma, hematocrit determinations, extrapolation of a dye-disappearance slope, and the application of a factor to correct for plasma trapped between centrifuged erythrocytes. The accurate determination of such small concentrations of dye in whole blood, however, approaches the limits of accuracy of the cuvette oximeter used.

LIMITATIONS OF METHOD

1. Determinations of cardiac output and Evans blue space, using an oximeter, cannot reliably be made on patients whose arterial blood does not become fully saturated with oxygen while they are breathing 100 per cent oxygen. The explanation lies in the fact that the blood used for making up known dilutions of dye must have the same degree of oxygen saturation that existed in the circulating arterial blood of the subject during the dye injection procedure; otherwise the calibration would be invalid for quantitative application to the dye curve. If one could be sure that there was no change in the degree of desaturation of the blood drawn to make up the known dilutions, it would be possible to make these quantitative determinations in patients with arterial oxygen desaturation; according to the technique used,

however, the likelihood of desaturated blood picking up oxygen from the air before the known dye dilutions are run through the oximeter is a distinct possibility. In cases where 100 per cent saturation is achieved, the fact that the control-blood sample of the dilution series absorbs essentially the same amount of red light as that absorbed by the subject's circulating, undyed arterial blood is evidence that significant desaturation of such blood does not occur on exposure to air at room temperature between the time the blood is drawn and the time it is again run through the oximeter with the dilution series.

2. Experience has shown that Evans blue dye, given intravenously in amounts exceeding 50 mg., is likely to cause a visible bluish discoloration of the subject. No other ill effects in human beings have been observed in more than 150 injections of the dye. The discoloration itself, however, is alarming to some and, if severe, may persist for several weeks. Since satisfactory dye curves in adults have rarely been obtained with doses of less than 20 mg., the procedure is not a suitable one for making repeated determinations within a short period of time.

3. A discussion of artefacts which may occur in some records appears elsewhere.[19] While artefacts do not constitute a true limitation of the method, failure to recognize them may result in erroneous measurements and conclusions. They may be produced by electrical or mechanical instability of the instruments, faulty injection or sampling procedures, or variations of a physiologic nature in the subject. Experience with the method will largely eliminate errors due to nonrecognition of artefacts. It is important to record, simultaneously with the dye curve, other physiologic variables such as heart rate and respiratory rate, in order to detect artefacts of a physiologic nature.

OTHER METHODS EMPLOYING A CONTINUOUS RECORDING TECHNIQUE

Two other procedures for calculating cardiac output by methods of recording continuously the changes in concentration of an indicator in flowing arterial blood have appeared recently.* The first of these, a conductivity method,[27] uses intravenously injected salt solution as the indicator. Impedance changes produced in arterial blood are picked up by electrodes inserted into the blood stream, and the pattern is recorded on a cathode-ray oscilloscope. The contour of the pattern during the first circulation of the indicator is similar to that of the dye curves previously described, and calculations are based on the same principle. The data presented were obtained from heart-lung preparations and intact dogs.

The second method,[28] which was published after the present studies were completed,[13] utilizes Evans blue dye and a photoelectric device to follow and record changes in the transmission of light through arterial blood at a wavelength of 628 mμ. Measurements and calculation of cardiac output are carried out in essentially the same manner as that set forth in this paper.

*A third study has been reported from Germany[29] since preparation of this article. The authors describe a method for recording time-concentration curves of intravenously injected saline solution plasma, or Evans blue dye from the exposed common carotid artery of the dog, using a selenium photocell with amplification.

A good correlation with determinations of cardiac output by the Fick principle is claimed for each of these methods. In the conductivity method this is achieved by applying a 10 per cent correction for the amount of salt presumably lost in the lungs on the first circulation, this being the magnitude of the average discrepancy between simultaneous determinations of output by direct measurement and by the conductivity method in heart-lung preparations. A similar correction of 8 per cent was found necessary for good correlation between the conductivity and the direct Fick methods in seven intact dogs. The use of an indictor which may readily diffuse from the blood stream makes the quantitative recovery and measurement of such an indicator difficult. The proponent mentions other problems of an electrical nature, apparently due to motion of the arterial wall supporting the electrodes. The fact that repeated determinations can be made at intervals as short as one minute is an attractive feature of the method.

The principle of the second method is essentially that of the present study, though the instruments differ. Only one wave band of light (visible red) is used in the photoelectric pick-up unit, so that no compensation can be made for the effect of changes in hemoglobin concentration or rate of blood flow on the absorption of red light by the blood. There is no mention made of steps taken to insure the constancy of oxygen saturation of the blood in which dye concentrations were being measured. From the published recordings, it appears that the instrument is sensitive to changes in intra-arterial pressure and rate of flow as well as to variations in light absorption due to the changes in oxygen saturation or concentration of the dye. A fair correlation with Fick determinations was achieved when the authors added a 6 per cent correction to the cardiac output as determined by the dye method to include coronary blood flow in their calculations. The necessity for this correction has not been established.

SUMMARY

A method has been described whereby changes in the concentration of intravenously injected Evans blue dye can be measured continuously in whole flowing arterial blood by the use of a simultaneous single-scale and double-scale oximeter. When blood was fully saturated with oxygen, the standard deviation of individual photoelectric determinations of dye in blood from an average calibration curve including blood samples from fifteen subjects was 6 per cent, no systematic variation with hemoglobin concentration being observed. This method has been applied to the determination of blood volume and cardiac output by the dye dilution procedure.

The standard deviation of 15 in vitro blood volume determinations was 5 per cent. Successive determinations of Evans blue space in twelve normal subjects approximately 90 and 360 seconds after injection of the dye showed average values of 65.4 ± 2.4 and 72 ± 3.0 ml. per kilogram, respectively.

The average cardiac index of ten normal "basal" subjects was 3.2 ± 0.2 liters per minute per square meter. Differences between direct Fick and dye determinations in 8 patients ranged from −17 to +23 per cent without apparent systematic deviation.

Use of an empirical correction and acceptance of a moderate increase in variability allow all measurements to be made directly from the photographic record. Further simplification of this method is thus attained by eliminating the necessity for a semilogarithmic replot of the photographed time-concentration data.

The authors acknowledge with gratitude the technical assistance of Roy Engstrom, Lucille Cronin, Rita Schmelzer, Ardyth Hanson, Janet Robinson, and Tom Bunting.

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A MODIFIED PROCEDURE FOR ASSESSING SEPARATE KIDNEY FUNCTION IN CHRONIC DOG PREPARATIONS WITH OBSERVATIONS ON THE EFFECT OF CHRONIC UNILATERAL VENOUS CONGESTION

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ANALYSIS of a single chronic experimental factor acting on the kidney, independently of other variables, is best performed with one kidney serving as a control to the changes provoked in the other kidney. This permits expression of results as a ratio eliminating nonspecific trends acting on both kidneys. Compensation by the control kidney permits specific analysis of changes in the experimental kidney without interference of general disturbance of homeostasis, i.e., electrolyte and waste retention.

The control kidney was used in acute experiments by Asher and Pearce,[1] who claim to be the initiators of this method. The same year Rowntree and associates[2] attempted chronic experiments on female dogs by ureteral catheterization through a cystoscope, whereas Hara, in 1922,[3] devised the first successful fistulization operation in Asher's laboratory. More recently, Selkurt and co-workers[4, 5] and Blake and associates,[6] used the control kidney in their investigations on hemodynamic factors of kidney function in acute experiments.

The problem of separate urine collections in chronic dogs has prompted development of various surgical techniques. Ureterostomies and fistulization operations with various cannulas have proved unsatisfactory.[7-9] Explantation of the ureters has been tried,[7, 8, 10] though only Grabfield and Swanson[11] report satisfactory results with this procedure. These difficulties induced Hara,[3] followed by Koennecke,[12] to develop a preparation in which the bladder was divided into separate pouches opening at the abdomen. Urine was collected by funnels on the erect animal. Few other investigators, if any, used this technique. Artificial exstrophy of the bladder, however, proposed in Abderhalden's Handbuch[8] and performed in this country by the Dragstedts,[13] Pilcher and associates,[14] and Maluf,[15] seems to be the operation of choice.

Recent experimental work on animals by various investigators[5, 6, 16, 17, 18] has renewed interest in the role of chronic venous stasis of the kidneys as a cause of edema in congestive heart failure. It was therefore proposed to assess chronic preparations allowing separate urine collections from explanted kidneys and kidneys left in situ for further study of the effects of unilateral venous congestion in the self-controlled conditions already mentioned.

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This investigation was carried out during the tenure of a Postdoctorate Fellowship of the United States Public Health Service as partial fulfillment of work in a Training Course for Cardiovascular Investigators (1950-51). The expenses for this investigation were defrayed by a grant from the American Heart Association, Inc., to Dr. C. J. Wiggers, Program Director.

Received for publication, June 29, 1951.

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MATERIALS AND METHODS

Procedures for Assessing Separate Kidney Function in Chronic Animals.—

Surgical Procedure: Separate ureterostomy to the abdomen was found to result in sloughing or late shrinkage of the circular skin scar with obstruction to the ureters. Therefore artificial exstrophy of the entire bladder was performed. At first the bladder was resected around the trigone as proposed by Maluf.[15] It was found, however, that shrinkage of the circular scar made separate urine collections difficult within two to three weeks after operation. For this reason the procedure was modified in that the whole bladder was grafted to the abdomen. Such preparations last without deterioration. The bladder was delivered through an abdominal midline incision extending to the pubic bone, in order to divide the urethra as distal from the bladder neck as feasible. The urachus and the peritoneal attachments of the

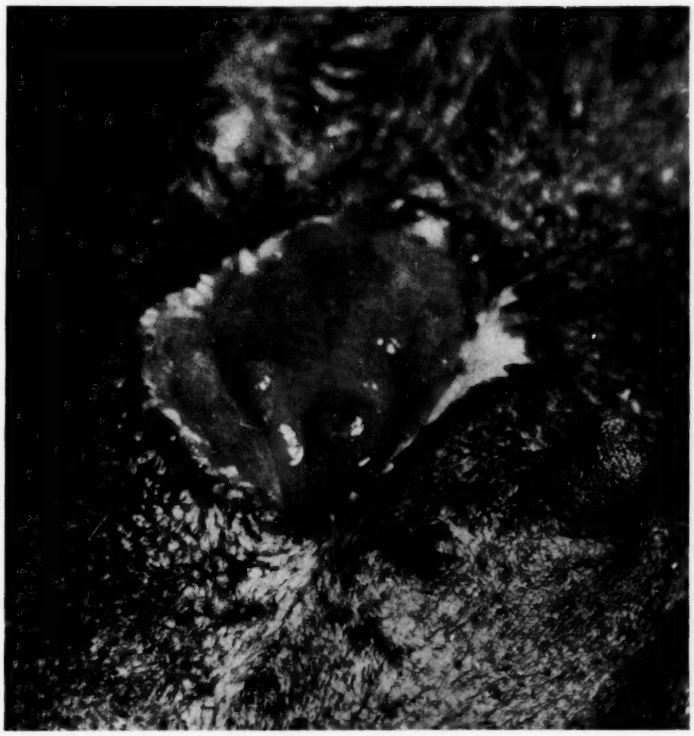

Fig. 1.—Bladder explant fifty days after operation showing both ureteral openings.

bladder and ureters were severed between double ligatures so as to mobilize the bladder sufficiently to avoid any traction when exteriorized. Peritoneum and fascia were closed down to an opening adequate in size for passage of the ureters, but small enough to avoid herniation. After ligature of the vessels running on its surface, the bladder was opened and its periphery anchored with separate stitches in the subcutaneous tissue after wide excision of a circular skin flap and undermining of the skin edges. No continuous suture was allowed for anchoring the bladder edge in the subcutaneous tissue, as this favored circular retraction of the flap. The end result is shown in Fig. 1.

Urine Collection and Clearance Techniques: Separate collections of urine from both ureters were obtained by use of a transparent plastic capsule 20 mm. in diameter, provided with a straight central partition, as shown in Fig. 2. This capsule is applied to the explanted

bladder so that the partition will fit between the ureteral openings (Fig. 3). Air admission orifices avoid pooling of urine. Through these, known volumes of fluid can be drawn to rinse the system and dye may be admitted after completion of the experiment to prove that no admixing occurs between the two sections of the cup. This instrument was devised because catheterization of the ureters or separate ureteral suction cups[15] were found to produce functional disturbances or collection artefacts. *Catheterization* of the ureters initiates reflexes conducive to oliguria, besides being a source of ascending infection; leakage occurs at the higher rates of urine flow. Others,[20] using the method of direct ureteral catheterization, found it necessary to obtain three-hour collections for assessing urine volume and sodium excretion with a margin of error too great for the purpose of the present investigation. *Separate suction cups* overlap if the ureteral orifices are close and cannot be applied securely. Bleeding and aspiration of the mucosa surrounding the ureters, creating unpredictable degrees of lateral obstruction to urine flow, is produced by small cups requiring strong suction for a firm hold on the bladder. Leakage occurs at peak diuresis, as there is inadequate capacity to accommodate the sudden spurts ejected by the ureters.

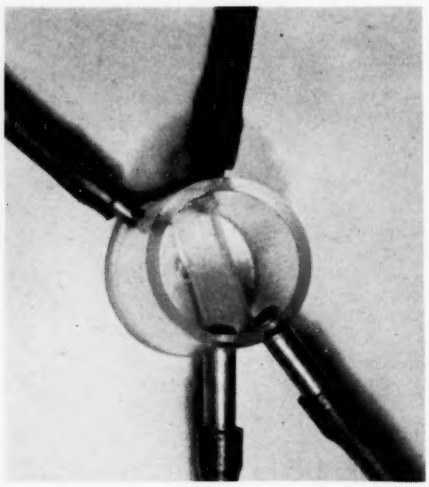

Fig. 2.—Plastic transparent capsule for separate urine collections. The open side faces upward to show the central partition. Lower tubes are suction outlets to graduate cylinders where urine is collected. Upper tubes are inlets for flushing and air admission through small openings in the tubing.

The relatively large surface of the capsule here described makes gentle suction adequate to hold it securely against the mucosa, permitting casual movements of the dog without interruption to the collection. The chambers are large enough to accept the spurts of urine at high urine flows, and dead space is avoided by constant admission of air through a small orifice in each inlet tubing.

Clearance experiments were performed on trained animals. Sufficient urine flows and sodium outputs were obtained with Ringer's solution given by venoclysis and adjusted to the animal's individual tolerance. Blood samples were taken from an indwelling jugular needle. A priming solution of 15 mg. creatinine per kilogram and 6 mg. p-aminohippurate (PAH) per kilogram was administered intravenously, while single subcutaneous injection of 0.2 Gm. creatinine per kilogram and 40 mg. PAH per kilogram insured adequate plasma levels of the test substances. Each experiment consisted of two to four urine collection periods of a duration of ten to fifteen minutes. Analysis for sodium concentrations were performed on a flame photometer with internal lithium standard. Urine and plasma were analyzed with standard methods for creatinine and PAH.[4]

Technique for Producing Chronic Unilateral Congestion of the Kidney.—Partial ligature of the inferior vena cava between the renal veins was adopted as the method for elevation of the venous pressure to the left kidney. The ligature was placed between the junctions of the left kidney. The ligature was placed between the junctions of the left and right renal veins with the inferior vena cava. This is made possible by the more caudad branching of the left vein in the dog. The right vein was permitted to empty into the inferior vena cava with minimal obstruction to the control kidney.

Surgical approach was either through a high midline abdominal incision with dissection performed on the right side of the mesentery after retraction of the intestinal loops to the left, or retroperitoneally through a right lumbar incision in the angle between the spine and the last rib. This latter approach entails the danger of pneumothorax and of a difficult dissection in the case of a high-lying venous junction. It was found that secure ligation around the vena cava with a No. 6 catheter in its lumen resulted in the degree of constriction necessary to elevate the distal pressure to +200 to +300 mm. saline after the catheter was withdrawn.

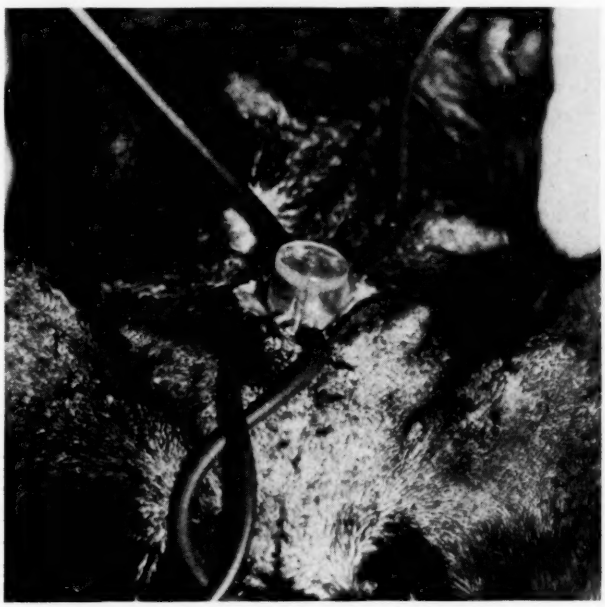

Fig. 3.—The collection capsule in place. Legend as in Fig. 2.

Inferior vena cava pressures were followed by repeated catheterization through branches of the femoral vein, and were read with reference to the level of the vena cava on a saline manometer connected to the venous catheter.

Congestion by partial venous ligature of the left kidney explanted in the flank* and separated from venous collateral pathways was attempted in order to obtain postconstriction venous pressure readings by transcutaneous puncture. Subsequent dissection of the vein imbedded in scar tissue proved difficult and the procedure was therefore abandoned.

RESULTS

Clearances were determined in six successfully operated dogs in which the ureteral openings were far enough apart to permit separate urine collections without intermixing or partial obstruction to one duct. Table I demonstrates

*The author is indebted to Dr. L. Peters, Dept. of Pharmacology, Western Reserve University, for demonstration of a modified Rhoads technique for explantation of the kidney.[19]

TABLE I. RESULTS OF CONTROL EXPERIMENTS ON SIX DOGS. EACH EXPERIMENTAL DATA REPRESENTS THE AVERAGE OF TWO TO FOUR URINE COLLECTION PERIODS OF TEN TO FIFTEEN MINUTES

DOG NO. AND WEIGHT	DATE OF EXPERIMENT	URINE VOLUME			CREATININE CLEARANCE GLOMERULAR FILTRATION RATE			P-AMINOHIPPURATE CLEARANCE RENAL PLASMA FLOW			FILTRATION FRACTION			SODIUM CLEARANCE		
		R	L	L/R	R	L	L/R	R	L	L/R	R	L	L/R	R	L	
		ML/MIN.			ML/MIN.			ML/MIN.			PER CENT			ML/MIN.		L/R
A. Animals with Kidneys in situ																
9	3/28	0.76	0.75	0.99	47.4	43.4	0.92	132.9	126.8	0.95	35.7	34.2	0.96	0.795	0.785	0.99
(23 kgs.)	4/2	0.85	0.86	1.01	44.4	42.6	0.96	106.1	105.2	0.99	41.8	40.5	0.97	0.875	0.925	1.06
10	4/21	0.65	0.66	1.01	34.0	33.7	0.99	105.8	101.3	0.96	32.2	33.3	1.03	0.207	0.197	0.95
(19 kgs.)	4/23	0.75	0.77	1.03	37.2	37.6	1.01	112.0	114.1	1.02	35.5	34.3	0.97	0.520	0.437	0.84
	4/24	0.59	0.60	1.02	37.8	37.7	1.00	122.9	119.2	0.97	30.9	31.7	1.02	0.463	0.469	1.01
11	4/30	0.73	0.79	1.08	64.1	63.4	0.99	173.2	173.7	1.00	37.0	36.5	0.99	0.676	0.512	0.76
(19.7 kgs.)	5/3	1.11	1.04	0.94	74.3	73.4	0.99	194.3	195.6	1.01	38.2	37.6	0.98	1.53	1.16	0.76
12	5/7	0.71	0.74	1.04	63.0	65.9	1.05	188.4	198.4	1.05	35.2	33.4	0.95	0.093	0.109	1.17
(24.5 kgs.)	5/8	0.83	0.88	1.06	62.9	60.8	0.97	163.0	166.2	1.02	38.8	36.8	0.95	0.141	0.154	1.09
B. Animals With Left Kidney Explanted																
6	2/8	0.17	0.17	1.00	52.3	48.7	0.93	110.0	117.0	1.06	47.5	41.6	0.87	0.166	0.168	1.01
(17 kgs.)	2/12	2.30	2.50	1.09	73.1	71.8	0.98	165.1	168.5	1.02	44.7	42.5	0.95	1.23	1.16	0.94
	2/15	1.80	2.00	1.11	54.9	51.7	0.94	150.9	146.4	0.97	36.5	35.2	0.96	1.64	1.62	0.99
8*	3/15	1.31	1.25	0.95	78.2	73.9	0.94	208.4	209.0	1.00	37.5	35.4	0.94	2.31	2.04	0.88
(18 kgs.)	3/19	0.53	0.54	1.02	85.3	72.0	0.84	174.1	153.1	0.88	49.8	47.5	0.95	0.887	0.685	0.77
	3/21	0.81	0.65	0.80	79.2	71.6	0.90	168.4	156.1	0.93	47.2	45.9	0.97	1.39	1.11	0.80

*In dog No. 8 an angiostomy cannula was located on the renal vein.

TABLE II. RESULTS OF FOLLOW-UP EXPERIMENTS ON DOG NO. 10, WITH THE LEFT KIDNEY UNDER CHRONIC STASIS

DATE OF EXPERIMENT	DAYS AFTER CONSTRICTION	VENA CAVA PRESSURE		URINE VOLUME			CREATININE CLEARANCE GLOMERULAR FILTRATION RATE			P-AMINOHIPPURATE CLEARANCE RENAL PLASMA FLOW			FILTRATION FRACTION			SODIUM CLEARANCE			LOAD REABSORBED	
		PROXIMAL	DISTAL	R	L	L/R	R	L	L/R	R	L	L/R	R	L	L/R	R	L	L/R	R	L
		MM. SALINE		ML/MIN.			ML/MIN.			ML/MIN.			PER CENT			ML/MIN.			PER CENT	
*Average of Controls Preceding Constriction**																				
–	–	+60† +215‡		0.66	0.68	1.03	36.4	36.3	1.00	113.6	111.6	0.98	32.9	33.1	1.01	0.39	0.36	0.92	98.89	98.97
Follow-up Experiments After Constriction																				
4/28	4	–	+275	0.77	0.56	0.73	30.9	27.0	0.87	121.1	83.2	0.69	25.6	32.5	1.27	0.072	0.051	0.71	99.75	99.80
5/2	8	+70	+195	0.61	0.53	0.87	34.3	31.4	0.92	116.2	92.9	0.80	29.5	33.8	1.15	0.359	0.266	0.74	98.90	99.11
5/10	16	+70	+180	0.95	0.84	0.88	34.7	33.5	0.97	139.3	115.6	0.83	24.9	29.0	1.16	0.598	0.483	0.81	98.16	98.46
5/17	23	–	+120	0.64	0.56	0.87	37.2	36.1	0.97	136.8	117.5	0.86	26.6	29.8	1.12	0.539	0.409	0.76	98.47	98.80
5/23	29	–	+175	1.19	1.02	0.86	38.6	38.2	0.99	149.3	128.5	0.86	27.4	30.1	1.10	0.974	0.753	0.77	97.35	97.93

*Individual values appear in Table I.
†Inferior vena cava pressure with open abdomen before constriction.
‡Inferior vena cava pressure with closed abdomen immediately after constriction.

that reasonably symmetric and consistent left to right ratios can be obtained in control experiments which assess urine flow, sodium clearance under saline load, effective renal plasma flow, and glomerular filtration rate. The values obtained on dogs No. 6 and No. 8 (Table I-B) show that kidney function remains fairly intact after explantation to the flank, whereas the entire set of results entered in Table I proves adequacy of artificial exstrophy of the bladder in conjunction with the urine collection method here proposed.

Table II illustrates on one dog the effects of chronic stasis in the left kidney by partial constriction of the inferior vena cava, expressed as ratios to the clearance values of the right kidney. Vena cava pressure was maintained about 100 mm. saline above control pressure, after an initial drop occurred between the fourth and eighth postoperative days. The creatinine clearance ratio, initially depressed to 0.87 when venous pressure was 275 mm. saline, returned to 0.97 sixteen days after constriction when the venous pressure had decreased to 180 mm. saline as the result of the development of collateral venous channels. The PAH clearance was decreased initially to 0.69 of the control, then gradually recovered as the venous pressure declined, but never to more than 0.86 of control. As a consequence the filtration fraction was continually elevated.

Sodium clearance decreased to 0.71 after ligation, but tended to remain depressed, the highest ratio observed during the period of elevated venous pressure being 0.81. Urine volume ratio, lowered four days after operation to 0.73, tended to recover better than sodium excretion, rising to an average ratio of 0.87 for the latter period of venous pressure elevation.

DISCUSSION

Artificial exstrophy of the bladder presents a number of problems dealt with in the description of the surgical technique. It suffices to say that the number of dogs in which the capsule cannot be applied (four of thirteen in our unselected series) because of extreme proximity of the ureters, might be reduced by using only squat dogs, weighing more than 20 kilograms.

The consistent results of the clearance experiments brought together in Table I testify to the adequacy of the surgical technique and the urine collection method here proposed for assessing separate kidney function in unanesthetized chronic dog preparations.

The data on the effect of chronic unilateral congestion on kidney function in one dog are entered in Table II.

The results on water and sodium excretion correspond to those found in one group of the acute experiments reported by Hall and Selkurt,[5] and also to the results of Blake and co-workers,[6] and Fishman and associates.[21] In these investigations and in the experiment here reported, a decrease of the values concerning those functions was found to occur without a significant drop in glomerular filtration rate, thus pointing to an effect of increased venous pressure on the tubular reabsorptive activity, independent of filtered water and sodium loads. To avoid repetition we refer to the discussion of possible mechanisms, correlation of these findings with recent clinical investigation, and bibliography contributed by the aforementioned authors. It may be added, however, as a point of direct

observation, that in the present experiment urine volume tended to recover more completely than sodium excretion, suggesting that specific sodium retention occurs as the result of venous pressure elevation. The trend in one of the above reports[5] points to the same effect.

The observed drop in plasma flow correlates well quantitatively with one group of data[16] obtained on dogs and with the clinical data of Bradley[22] and Maxwell and associates,[23] but not with those of Blake and co-workers,[6] who reported no change in flow at comparable levels of venous pressure. It is assumed that PAH excretion in these experiments is not impaired, for it has been shown by Bradley[22] that there is no reduction of PAH extraction with similar venous pressure elevation.

The drop in plasma flow, as against lesser decrements in glomerular filtration rate, results in elevation of the filtration fraction which can be interpreted in terms of Ludwig's[24] original postulate, demonstrated by Winton,[25] that venous obstruction elevates filtration pressure. In Winton's elaboration, this increase in filtration pressure should logically result in elevation of the filtration rate if it were not for the obstruction to urine flow in the tubules set up by the intrarenal congestion. The experiment here reported differs from the findings in acute animal experiments[16] and clinical investigation,[22, 26] where filtration decreased with plasma flow, the filtration fraction thus remaining unchanged. These differences may depend on the degree of intratubular obstruction produced by the experimental congestion of the kidney.

From this preliminary observation it is concluded that intrinsic renal readjustment to venous stasis is not complete and that the thorough recovery of water and Na excretion observed by Hwang and associates[17] is due to the systemic effect on kidney function elicited by the retention of electrolytes due to the ligature of the inferior vena cava proximal to both kidneys. Similar recovery has been noted by Mueller and co-workers[20] when both renal arteries were constricted, but not if only one artery was subjected to this procedure.

SUMMARY

Chronic preparations for comparison of control to experimental kidney by clearance techniques were obtained by explanting the whole bladder to the abdomen of female dogs and collecting separate urines by suction applied to a plastic capsule provided with a central partition. Symmetric and consistent kidney function, assessed by urine flow, p-aminohippurate clearance, creatinine and sodium clearances, as left to right experimental ratios, demonstrated adequacy of the method.

Partial ligature of the inferior vena cava between the renal veins was adopted for the study of the intrinsic effects of chronic elevation of venous pressure on the kidney. The technique is described and the preliminary results obtained are discussed. Glomerular filtration is little influenced by the procedure, whereas sodium retention, accompanied by a lesser retention of water, occurs, pointing to an effect of increased venous pressure on the tubular reabsorption of sodium and water, independent of the filtered loads. The decrease in renal plasma flow against maintained filtration rate results in increase of the filtration fraction.

I express my appreciation to Dr. E. E. Selkurt for direction in this investigation, and to Dr. C. J. Wiggers for advice and help in the project and preparation of the manuscript. Also the opportunity is taken to thank Dr. E. R. Ritter for valuable assistance in these experiments.

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HUMAN SERUM TRIBUTYRINASE

III. Levels in Cancer

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ABNORMAL variations in serum lipolytic enzymes, associated with a number of disease states, have been found by several workers. Hangleiter and Reuter[1] reported decreased serum lipase levels in patients suffering from tuberculosis, cancer, and diseases of the liver and the gall bladder. Ingelfinger[2] found high serum lipase and normal serum amylase activities in cancer of the pancreas. Faber[3] studied variations in serum cholinesterases under normal and pathologic conditions in 400 persons, and found particularly low values in liver diseases, anemia, undernutrition, and cancer. He stated that the determination of the cholinesterase level by itself is of no diagnostic value, but may contribute to the discovery of some general factor affecting the serum enzyme content. However, Lagerlöf[4, 5] was unable to find any abnormal variations in serum esterases in a number of disease states, including those affecting the pancreas, biliary ducts, and liver. Pegni and Meoni[6] stated that the lipolytic potency of blood varied widely in the same person at short intervals, and that such determinations are of no value for prognosis. Tuba and Hoare[7] noted significant decreases in the serum tributyrinase activity of patients suffering from advanced tuberculosis.

METHODS AND MATERIALS

Samples of blood were obtained from patients attending the Edmonton Cancer Clinic, and a few samples from proved malignant cases were received from the Calgary General Hospital. Essential clinical information was eventually obtained in all cases. Serum was always separated immediately after the arrival of the sample in the laboratory. The method for the determination of tributyrinase activity has been described in a previous paper.[8]

RESULTS

Serum tributyrinase activity was determined for 300 Cancer Clinic patients. A comparison of tributyrinase levels in 280 normal[7] and in 300 Cancer Clinic subjects is made in Table I. The mean value for the latter group is only 9 per cent below the normal, but the difference between means is highly significant. Since final diagnoses on several cases were not complete at the time of tributyrinase determinations, the difference from the normal is not so readily apparent for the total Cancer Clinic group as it is for the fifty proved malignant cases, which have a mean value 34 per cent below the normal ($t = 13.0$; t at 1 per cent level = 2.58).

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Assisted by grants from the Alberta Tuberculosis Association, and the Committee on Allocation of Medical Research Grants, University of Alberta.
Received for publication, July 2, 1951.

TABLE I. SERUM TRIBUTYRINASE LEVELS IN NORMAL SUBJECTS AND CANCER CLINIC PATIENTS

	NUMBER OF SUBJECTS	MEAN TRIBUTYRINASE LEVEL (UNITS/100 ML.)	STANDARD DEVIATION	STANDARD ERROR OF THE MEAN
Normal				
Total	280	403	± 89	±5.0
Males	174	417	± 77	±5.8
Females	106	379	± 75	±7.4
Cancer Clinic				
Total	300	365	± 97	±5.6
Males	152	355	±114	±9.3
Females	148	377	± 70	±5.8
Known malignant cases	50	266	± 64	±9.0

Comparison of means for normal and cancer clinic subjects: $t = 2.57$ at 1% level; $t = 4.9$, highly significant.

Results for some of the various types of suspected cancer cases are presented in Table II. It is interesting to note that subnormal mean values were found in the serum of patients suspected of cancers of the gastrointestinal tract, prostate, and bladder. Serum from patients with possible cancers of the breast, cervix, lung, and skin, showed little difference from the normal mean values.

TABLE II. SERUM TRIBUTYRINASE LEVELS IN 205 CANCER CLINIC PATIENTS SUSPECTED OF VARIOUS TYPES OF CANCER

SUSPECTED CONDITION	NUMBER OF CASES	MEAN TRIBUTYRINASE LEVELS (UNITS PER 100 ML.)	S.D.	S.E.
Cancer of breast	49	396	± 83.4	± 12.0
Cancer of cervix	23	411	± 56.9	± 12.1
Cancer of lung	9	358	± 76.6	± 27.1
Cancer at head of pancreas	3	296	± 47.4	± 33.6
Cancer of prostate	5	263	± 43.8	± 21.9
Epidermoid cancers	44	377	± 86.3	± 13.2
Cancer of colon	11	449	± 80.6	± 25.5
Cystic disease of bone	4	493	±112.0	± 65.9
Cancer of bladder	3	346	±167.0	±119.0
Cancer of stomach	37	334	± 51.4	± 8.6
Cancer of rectum	17	322	± 62.7	± 15.7

This finding is more strikingly evident in Table III where a differentiation has been made between proved malignant and nonmalignant or normal conditions. As in our preceding paper,[7] the serum tributyrinase values were classified as follows:

Normal	360-550 units per 100 ml.
Low normal	310-360 units per 100 ml.
Subnormal	210-310 units per 100 ml.
Very low	below 200 units per 100 ml.

The great majority of cases of cancer of the stomach, colon, rectum, bladder, prostate, and at the head of the pancreas showed subnormal or very low tributyrinase activity and in only one case was a normal value found for

this enzyme. Normal levels were obtained in nonmalignant cases. Cancers of the breast, cervix, skin, and cystic disease of bone showed a normal tributyrinase level.

SUMMARY

Serum tributyrinase levels have been determined in 300 Cancer Clinic patients. This group showed a significantly lower mean value than a group of normal subjects.

TABLE III. TRIBUTYRINASE LEVELS IN MALIGNANT AND NONMALIGNANT CASES

CONDITION	TRIBUTYRINASE (UNITS PER 100 ML. SERUM)			
	CASE NUMBER	MALIGNANT	CASE NUMBER	NONMALIGNANT
Cancer of stomach	1	350	14	444
	2	288	15	368
	3	321	16	616
	4	332	17	504
	5	292	18	440
	6	308	19	420
	7	288	20	452
	8	292	21	460
	9	144	22	396
	10	164	23	420
	11	196	24	400
	12	92		
	13	208		
Cancer of colon	25	340	30	348
	26	136	31	412
	27	288	32	548
	28	260		
	29	280		
Cancer of rectum	33	240		
	34	328		
	35	340		
	36	376		
	37	216		
	38	320		
	39	264		
Cancer of bladder	40	172	42	528
	41	308		
Cancer of prostate	43	240		
	44	198		
	45	272		
	46	292		
	47	216		
Cancer at head of pancreas	48	244		
	49	340		
	50	304		
Cancer of breast	51	428		
	52	392		
	53	408		
	54	400		
Cancer of cervix	55	348	59	436
	56	360	60	416
	57	376	61	324
	58	504	62	412
Skin cancers	63	368		
	64	352		
	65	456		
Cystic disease of bone	66	644		
	67	420		
	68	508		
	69	400		

Of a total of thirty-five patients suffering from cancers of the stomach, colon, rectum, bladder, head of pancreas, and prostate, only one patient had serum tributyrinase activity in the normal range; eight were in the low normal range, and twenty-six were in the subnormal or very low range. Seventeen patients with other malignant conditions were in the normal range.

It is felt that further investigations should be carried out to establish the significance of these findings as a possible aid to early diagnosis of certain types of cancer.

We are very grateful to Dr. Victor Wright, Director of Cancer Clinics for Alberta, for his assistance in providing samples and information. Thanks are also due to Dr. Lola McLatchie, pathologist at the Calgary General Hospital, and to Miss Betty Alexander, technician.

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TREATMENT OF CHRONIC TYPHOID CARRIERS WITH TERRAMYCIN*

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CHRONIC typhoid carriers are a danger to their families and to the public, and, in spite of strict supervision, occasionally infect others. Although their numbers have decreased over the years, there are still 422 known carriers residing in New York State exclusive of New York City, and they obviously present a problem. A means of cure would be a great boon to the public and would be welcomed by the carriers themselves.

Cholecystectomy cures around 68 per cent of biliary carriers.[1] However, surgery is contraindicated in many because the average carrier is elderly and a poor surgical risk. In some instances, permission for operation is refused, and in others, cholecystectomy is not indicated because the focus of infection is not in the gall bladder. Thus, this operation offers only a limited benefit.

Medical treatment of the carrier state has been disappointing.[2] The literature is filled with instances where cure has been reported as due to one drug or another, only to be followed by additional reports failing to confirm the original observation. The recent paper by Vaichulis and his associates[3] citing cures of gall bladder and other carriers by the use of antagonistic bacilli or a combination of drugs is one that needs to be confirmed.

The action of terramycin against *Salmonella typhosa* in vitro and in vivo in mice[4] and its ready excretion in the bile[5] led us to test its value in twelve chronic

TABLE I. TYPHOID CARRIERS IN THE PRESENT STUDY

CARRIER	AGE	SEX	HISTORY OF TYPHOID FEVER (YR. BEFORE)	BACT. PROVED DURATION OF CARRIER STATE (YEARS)	LENGTH OF RESIDENCE MENTAL HOSPITAL (YEARS)	VI AGGLUTININ TITER	BACTERIOPHAGE TYPE
1	76	M	No	5	1	Neg.	A
2	55	F	No	10	22	1:20	F
3	74	M	No	10	11	Neg.	Could not determine
4	74	F	No	10	30	Neg.	Could not determine
5	80	F	No	11	31	Neg.	Not done
6	40	F	4	4	1	1:40	E_1
7	71	F	No	6	22	1:20	E
8	55	F	11	9	11	1:10	"Imp. V"
9	55	F	No	6	16	Neg.	A
10	64	F	42	12	14	1:10	B_2
11	62	F	No	6	16	Neg.	"Imp. V"
12	67	F	13 (Ques.)	6	16	1:20	"Imp. V"

From the Bureau of Epidemiology and Communicable Disease Control, New York State Department of Health.
Received for publication, June 8, 1951.
*Terramycin was supplied through the courtesy of Charles Pfizer & Co., Inc.

TABLE II. TYPHOID CARRIERS. RESULTS OF STOOL EXAMINATIONS

CASE NO.	TERRAMYCIN 1.25 GM. DAILY																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1			−	?		?	?	?	?	?	?				?		?					?		?
2			?	?	?	?				?	?					?			+				+	
3			?	?		?	?	?	?	?	?						?					?		+
4		+	+	−	?	−				?	?					?			+			+		
5		−	?	?					?	?	?					?							?	
6		+	−	−	−	−	?		?	?	?					?			+				+	
7		+	?	?	?	?			?	?	?	?	?					?		?			?	
8		+	+	+	?	?			?	?	?	?	?					?		?			+	
9		+	+	?	?	?			?	?	?	?	?					?		+			+	
10		+	?	?	?	?			?	?	?	?	?					+		+			+	
11		+	?	?	?	?			?	?	?	?	?					+		+			+	
12		−		−	?				?	?	?	?	?					?		?			?	

typhoid carriers. Unfortunately, it proved ineffective in eradicating the carrier state. Nevertheless, the experience seems worth recording.

MATERIALS AND METHODS

Twelve chronic typhoid carriers, patients in two mental hospitals,* were studied. Pertinent data concerning these individuals are shown in Table I. Each of the patients had had many stools positive for *S. typhosa* since the carrier status had been discovered, and its persistence was proved in each carrier by demonstrating *S. typhosa* in each of three stools taken during the week before treatment was begun.† Typhoid Vi agglutinin studies were carried out with the thought that these might serve as an additional measure of cure, if the stools became negative for *S. typhosa* as a result of therapy. Previous gall bladder studies had shown many of these carriers to be harboring gallstones.

The dosage of terramycin hydrochloride used was 1.25 Gm. by mouth every six hours for ten days. In one hospital it was administered in capsules, in the other the capsules were emptied into a glass of milk because certain patients could not be induced to swallow capsules. Treatment was uneventful in all patients, except that a few vomited once or twice and one had diarrhea for two days.

RESULTS

In no instance was the carrier state permanently eliminated. Table II lists the results of fecal examination by day of collection following initiation of treatment. *Salmonella typhosa* were found in the stools of all carriers at some time during the period nine to thirty-six days after the cessation of treatment. The detection of the organism was made difficult or impossible during and shortly after treatment by the appearance in the stools of large numbers of organisms resembling members of the proteus family. The duration of this change in the intestinal flora is indicated in Table II by the distribution of reports recorded as of "questionable significance."

SUMMARY

Twelve chronic typhoid carriers treated with terramycin hydrochloride in a dosage of 1.25 Gm. by mouth every six hours for ten days persisted in excreting *S. typhosa* in their stools after the completion of treatment.

*Willard State Hospital and Harlem Valley State Hospital.

†All laboratory work done by Division of Laboratories and Research, New York State Department of Health.

BY DAYS FOLLOWING INITIATION OF TREATMENT WITH TERRAMYCIN

DAY FOLLOWING INITIATION OF TREATMENT																							
25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51+
				?								−						+							+	
−							+							+							+					
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\+ Stool positive for *S. typhosa*.

− Stool negative for *S. typhosa*.

? No *S. typhosa* found. Results of questionable significance because of presence of large numbers of organisms resembling members of the proteus group.

*Case 5—stool positive on fifty-third day.

**Case 12—stool negative on seventy-fourth day; negative on eighty-first day; positive on ninety-fourth day.

The authors wish to acknowledge the cooperation of Dr. Kenneth Keill, Director, Willard State Hospital, and Dr. Leo O'Donnell, Director, Harlem Valley State Hospital, in making these studies possible.

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COMPARISON OF IN VITRO SENSITIVITY OF FRESHLY ISOLATED PATHOGENIC BACTERIA TO TERRAMYCIN AND OTHER ANTIBIOTICS

(AUREOMYCIN, CHLORAMPHENICOL, PENICILLIN, AND STREPTOMYCIN)

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FOLLOWING the original report of Finlay and associates,[1] the antibacterial spectrum of terramycin has been investigated by several workers.[2-5] Comparative bacterial sensitivities to aureomycin and terramycin have been reported by Hobby and associates,[2] and by Bliss and co-workers.[4-5] Most of the organisms studied were stock laboratory strains. The similar activity of these antibiotics has been noted.

An extensive survey of the sensitivity of pathogenic bacteria to antibiotics was begun by Finland and co-workers before terramycin became available,[6-13] and in later papers they included data on in vitro activity of this drug too.

The present investigation was undertaken to determine the sensitivity to terramycin and other antibiotics of bacteria freshly isolated from hospital patients. Each strain was simultaneously tested with four or five antibiotics, penicillin being omitted for gram-negative bacilli. An attempt was made to determine which antibiotics had been administered prior to culture, with the thought that this might explain drug resistance.

MATERIALS AND METHODS

Agents.—Aureomycin hydrochloride was available in sterile 100 mg. vials.* Chloramphenicol in unsterile crystalline form was used.† The potassium salt of crystalline penicillin G in sterile vials containing 500,000 units,‡ and streptomycin calcium chloride complex in sterile vials containing the equivalent of 1 Gm. streptomycin base were used.§ In early experiments, unsterile terramycin hydrochloride powder|| with activity of 900 μg per milligram in terms of terramycin base was employed. In later experiments, sterile terramycin hydrochloride|| with activity of 915 μg per milligram was used.

Preparation of Solutions.—Standard solutions were prepared in concentrations of 400 units per milliliter for penicillin and 400 μg per milliliter for the other antibiotics using distilled water, except for chloramphenicol and the first terramycin standard. The chloramphenicol

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Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication, April 5, 1951.

*Supplied by Dr. Stanton M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

†Supplied by Dr. John Ehrlich, Parke, Davis & Company.

‡Commercial preparation of Schenley Laboratories, Inc.

§Commercial preparation of Sharp & Dohme, Inc., or of Merck and Company, Inc.

||Supplied by Dr. Gladys L. Hobby, Chas. Pfizer & Company.

col and, at first, the terramycin standard, were prepared in sterile "Penassay broth" (Difco) and sterilized by slow filtration through a No. 6 Seitz EK filter sheet. Subsequently, dilution standards were made from 1 Gm. vials of sterile terramycin hydrochloride provided by Dr. Hobby. Comparison of results in sensitivity tests simultaneously performed with the weighed, Seitz-filtered standard and the dilution standard showed no appreciable loss of activity on Seitz filtration.

The standard solutions were stored at −15° to −20° C. More concentrated standards were similarly prepared and stored for studying resistant strains, except for terramycin which tended to precipitate from concentrated solutions after freezing. Terramycin standard for resistant strains was freshly prepared in a concentration of 800 μg per milliliter, and serial dilutions were made immediately before use. There was no indication of loss of activity of any of the antibiotics during storage in the frozen state.

Method of Determining Sensitivity.—A twofold serial dilution tube method[14] was used. Each tube contained 0.5 ml. of antibiotic dilution in broth. The inoculum consisted of 1.5 ml. of 1:100 or, for some gram-negative bacilli yielding a heavy growth, of 1:200 dilution in broth of a twenty-four hour culture. "Penassay broth" (Difco) was used in all tests except where otherwise indicated in the tables. The final antibiotic concentrations ranged from 100 to 0.37 μg per milliliter for gram-negative bacilli, and from 25 to 0.08 μg per milliliter for gram-positive cocci. Fractions are smoothed off in the tables to read 3.1, 1.6, 0.8, 0.4, 0.2, and 0.1 μg per milliliter. Each strain was tested against all the antibiotics simultaneously, but resistant strains were retested with stronger concentrations, using only the agents for which end points were not reached. The tubes were incubated at 37° C. and read at twenty-four and forty-eight hours, the sensitivity being expressed as the lowest concentration of antibiotic which showed no visible turbidity. Tubes with antibiotic-free media were used as controls.

The sensitivities listed in the tables are on the basis of twenty-four hour readings. Forty-eight hour readings almost invariably showed a two- or fourfold increase in concentrations of terramycin and aureomycin needed to inhibit growth. With chloramphenicol, penicillin, and streptomycin the twenty-four and forty-eight hour readings were usually the same, although an occasional twofold increase was noted at forty-eight hours, especially with resistant organisms. In interpreting the results as to their bearing on human drug therapy, one should bear in mind the greater loss of activity of aureomycin solutions over a twenty-four hour period than of terramycin.

Organisms Tested.—Most of the bacteria studied were freshly isolated from hospital patients, although a few pseudomonas strains had been kept several months in a deep freeze.* The organisms were supplied to us at random without regard to resistance or past therapy. Additional strains were provided from stock cultures of the Communicable Disease Center, U. S. Public Health Service, Chamblee, Georgia. From Dr. Martha K. Ward, Special Bacteriology Unit, 10 strains of the tribe *Mimeae*[15] were obtained. To our knowledge, sensitivity studies on this group of organisms have not previously been reported. Six strains of shigella were obtained from the Enteric Bacteriology Laboratory through Dr. P. R. Edwards and Dr. William H. Ewing, who assisted us with serological identification of shigella and salmonella strains.

RESULTS

The results of sensitivity tests are shown in Tables I to V. These findings confirm previous reports that the antibacterial spectra of terramycin and aureomycin are generally similar. Both drugs inhibited a wide variety of gram-negative bacilli and appeared more active in vitro against gram-positive cocci than did chloramphenicol, except against resistant staphylococcus strains.

Terramycin, aureomycin, and chloramphenicol were effective against *E. coli*, with minor strain variations (Table I). Terramycin seemed to be the most

*The hospital strains were isolated and identified by Miss Dorothy N. Sage and Mr. James A. Burroughs of the Clinical Laboratory, Lawson Veterans Administration Hospital.

TABLE I. ESCHERICHIA COLI, AEROBACTER AEROGENES, ALCALIGENES, AND BRUCELLA

STRAIN	SOURCE	ANTIBIOTICS* BEFORE CULTURE	SENSITIVITY, μG/ML.			
			TERRAMYCIN	AUREOMYCIN	CHLORAMPHENICOL	STREPTOMYCIN
E. coli						
1.	Urine	None	1.6	12.5	6.25	25
2.	Peritoneum	None	1.6	6.25	6.25	25
3.	Urine	None	3.1	6.25	6.25	25
4.	Urine	None	3.1	6.25	6.25	25
5.	Urine	P	3.1	12.5	6.25	25
6.	Sputum	None	6.25	12.5	6.25	25
7.	Urine	A, P, S	6.25	25	6.25	12.5
8.	Bronchial washings	None	12.5	25	6.25	25
9.	Blood (p-m.)	P, S	12.5	25	12.5	12.5
10.	Urine	A, P, T	12.5	25	100	>100
A. aerogenes						
1.	Urine	P, S	3.1	25	50	>100
2.	Urine	P	3.1	50	50	>100
3.	Pleural fluid	S	6.25	12.5	50	6.25
4.	Feces	None	6.25	25	25	6.25
5.	Urine	A, C	6.25	50	12.5	>100
6.	Urine	P	12.5	50	12.5	6.25
7.	Lung	None	12.5	25	25	>100
8.	Urine	A, C, P, S	12.5	50	100	100
9.	Urine	P	25	50	>100	>100
10.	Urine	A, C, P, S, T	25	100	100	>100
Alc. fecalis						
1.	Urine	A, P, S, T	3.1	1.6	12.5	>100
2.	Urine	P	3.1	1.6	100	>100
3.	Urine	C, P, S	25	100	>100	>100
Brucella suis						
1.	Blood	P	<0.4	<0.4	1.6	<0.4
2. (same patient)	Blood	P, T	<0.4	<0.4	1.6	1.6
3.	Blood	P	0.8†	0.8†	3.1†	0.8†
4. (same patient)	Blood	P, T	<0.4	<0.4	3.1	3.1
5.	Blood	T	<0.4	<0.4	1.6	3.1

*A, aureomycin; C, chloramphenicol; P, penicillin; S, streptomycin; T, terramycin.
†Tested in tryptose phosphate broth.

active against *Aerobacter aerogenes.* All three of the newer antibiotics were very effective against two strains of *Mima polymorpha* and one of *Colloides anoxydana,* while seven strains of *Herellea vaginicola* and two of three strains of *Alc. fecalis* were sensitive to terramycin and aureomycin but resistant to chloramphenicol (Tables I, II). In contrast, terramycin and chloramphenicol appeared quantitatively more active against shigella than did aureomycin and streptomycin (Table II). Terramycin, in general, inhibited *Ps. aeruginosa* in lesser concentrations than did the other antibiotics (Table III). One strain was inhibited by terramycin, 3.1 μg per milliliter before treatment, while a second culture obtained after three days of therapy required 12.5 μg per milliliter for inhibition. Pseudomonas persisted in this patient's urine, but no further change in sensitivity occurred after ten days of treatment.

Ten proteus strains were resistant to terramycin, aureomycin, and streptomycin (Table III). In general, they required lesser concentrations of chloramphenicol for inhibition, but only three were inhibited by as little as 12.5 μg per milliliter.

Three strains of *Brucella suis* from different patients, but possibly from the same animal source, were quite sensitive in vitro to terramycin, aureo-

TABLE II. MIMEAE, SHIGELLA, SALMONELLA AND KLEBSIELLA

ORGANISM	SOURCE	ANTIBIOTICS* BEFORE CULTURE	SENSITIVITY, μG/ML.			
			TERRAMYCIN	AUREOMYCIN	CHLORAMPHENICOL	STREPTOMYCIN
Mimeae						
1. *Mima polymorpha*	Stock	Unknown	0.8	1.6	3.1	>100
2. *Mima polymorpha*	Stock	Unknown	0.8	0.8	0.8	>100
3. *Colloides anoxydana*	Stock	Unknown	0.8	0.8	1.6	6.25
4. *Herellea vaginicola*	Stock	Unknown	0.8	0.8	50	>100
5. *Herellea vaginicola*	Stock	Unknown	1.6	0.8	100	100
6. *Herellea vaginicola*	Stock	Unknown	1.6	0.4	100	50
7. *Herellea vaginicola*	Stock	Unknown	1.6	0.8	100	6.25
8. *Herellea vaginicola*	Stock	Unknown	3.1	1.6	100	50
9. *Herellea vaginicola*	Stock	Unknown	3.1	1.6	100	100
10. *Herellea vaginicola*	Stock	Unknown	3.1	1.6	100	>100
Shigella						
1. *Sh. flexneri* 3	Stock	Unknown	1.6	12.5	3.1	12.5
2. *Sh. flexneri* 2a	Stock	Unknown	1.6	12.5	1.6	6.25
3. *Sh. flexneri* 6	Feces	P	1.6	25	3.1	12.5
4. *Sh. dysenteriae* 2	Stock	Unknown	1.6	6.25	1.6	12.5
5. *Sh. dysenteriae* 2	Stock	Unknown	3.1	12.5	1.6	12.5
6. *Sh. boydii*	Stock	Unknown	3.1	12.5	3.1	25
7. *Sh. flexneri* 2	Feces	None	3.1	6.25	1.6	12.5
8. *Sh. sonne*	Feces	None	3.1	12.5	3.1	12.5
9. *Sh. sonne*	Feces	None	3.1	12.5	6.25	25
10. *Sh. sonne* I	Stock	Unknown	12.5	25	6.25	25
11. *Sh. flexneri* 1b	Stock	Unknown	12.5	12.5	3.1	25
Salmonella						
1. *Sal. typhimurium*	Blood	None	6.25	12.5	6.25	100
2. *Sal. typhimurium*	Feces	None	6.25	12.5	6.25	100
3. *Sal. oranienburg*	Feces	A, T	25	25	12.5	100
Klebsiella						
1. *K. pneumoniae* B	Bronchial washings	P	6.25	25	25	6.25
2. *K. pneumoniae* B	Sputum	None	12.5	50	12.5	6.25
3. *K. pneumoniae* B	Sputum	P	12.5	25	---	12.5

*A, aureomycin; P, penicillin; T, terramycin.

mycin, chloramphenicol, and streptomycin (Table I). There was no increase in resistance to terramycin when the organisms were recovered from patients in relapse eight to thirteen and one-half weeks after completion of treatment with 30 to 38 Gm. of terramycin, administered over a fifteen- to twenty-day period. Three strains of *K. pneumoniae* were inhibited by somewhat lesser concentrations of terramycin and streptomycin than of aureomycin (Table II).

Wide strain variations in sensitivity to streptomycin were noted with *E. coli, A. aerogenes, Ps. aeruginosa,* and members of the tribe *Mimeae,* but three strains of *Alc. fecalis,* three salmonella strains, and ten proteus strains were uniformly streptomycin-resistant.

Among gram-positive cocci, two strains of pneumococcus and several miscellaneous streptococci were highly sensitive to penicillin, terramycin, and aureomycin (Table V). Slightly greater concentrations of chloramphenicol were needed to inhibit these. Enterococci were considerably more resistant to penicillin than other streptococci, but eight of ten strains were inhibited by 6.25 μg per milliliter or less of terramycin or aureomycin (Table V). Larger concentrations of chloramphenicol were similarly required for inhibition of enterococci, all of which were also resistant to streptomycin. One strain re-

TABLE III. PSEUDOMONAS AERUGINOSA AND PROTEUS

STRAIN	SOURCE	ANTIBIOTICS* BEFORE CULTURE	SENSITIVITY, μG/ML. TERRAMYCIN	AUREOMYCIN	CHLORAMPHENICOL	STREPTOMYCIN
Pseudomonas						
1.	Urine	A, C, P, S	3.1	25	100	>100
	Urine	Same plus T, 2g/d, 3d	12.5	100	>100	>100
2.	Feces	A	3.1	3.1	50	12.5
3.	Urine	C, P, S	6.25	25	—	> 25
4.	Urine	P, S	6.25	25	50	>100
5.	Urine	P	6.25	50	100	>100
6.	Urine	C, P	6.25	50	>100	>100
7.	Urine	P, S	6.25	12.5	>100	>100
8.	Pleural fluid	A, P, S	6.25	50	50	>100
9.	Urine	P, S	6.25	50	>100	>100
10.	Leg ulcer	None	12.5	100	>100	25
11.	Urine	P	12.5	50	>100	50
12.	Urine	C, P, S	12.5	100	>100	>100
13.	Urine	C, P, S	12.5	50	>100	>100
14.	Urine	P, S	12.5	50	100	>100
15.	Urine	P, dihydro-S	12.5	100	>100	>100
16.	Pleural fluid	A, C, P, S	12.5	50	50	>100
17.	Urine	A, P, S	25	100	>100	>100
18.	Urine	C, P, S	25	100	>100	>100
19.	Sputum	P, T, 2g/d, 7d	25	100	>100	100
20.	Urine	C, P	50	>100	>100	6.25
21.	Urine	A, C, P, S, T, 2g/d, 1 d	50	>100	>100	>100
Proteus sp.						
1.	Pus	C, P, S	50	25	12.5	50
2.	Urine	A, P	100	100	50	100
3.	Urine	None	100	100	50	50
4.	Pus	P, S	100	100	25	50
5.	Urine	A, C, P, T, 3g/d, 8d	>100	100	50	50
6.	Urine	P, S	>100	100	12.5	>100
7.	Urine	P, T, 3g/d, 9d	>100	100	12.5	50
8.	Urine	A, P	>100	100	50	100
9.	Feces	None	>100	100	25	50
10.	Urine	A, P, T, 1g/d, 18d	>100	>100	50	25

*A, aureomycin; C, chloramphenicol; P, penicillin; S, streptomycin; T, terramycin.

quired 100 μg per milliliter of terramycin for inhibition. The patient from whom this organism was recovered had received terramycin five months before culture was taken.

Interesting variations in sensitivity were encountered among staphylococci (Table IV). Although ten of fourteen strains of *Staph. aureus* were sensitive to 3.1 μg per milliliter or less of terramycin, four strains were highly resistant, requiring 200 or more μg per milliliter for inhibition. These results paralleled those obtained with aureomycin except that the amounts of aureomycin needed to inhibit resistant strains were smaller. The four terramycin-resistant strains were also highly resistant to penicillin and moderately or highly resistant to chloramphenicol and streptomycin. On the other hand, four additional strains were highly resistant to penicillin but sensitive to terramycin and aureomycin.

Five of ten *Staph. albus* strains were extremely resistant to terramycin, requiring 200 or more μg per milliliter for inhibition (Table IV). These strains were also resistant to aureomycin, streptomycin, and penicillin. Two were quite resistant to chloramphenicol. An additional strain was highly resistant to penicillin but sensitive to terramycin, aureomycin, and chloramphenicol.

TABLE IV. STAPHYLOCOCCUS

ORGANISM	SOURCE	HEMO.	COAGULATION	ANTIBIOTICS* BEFORE CULTURE	SENSITIVITY, μG./ML.				
					TERRAMYCIN	AUREOMYCIN	CHLORAMPHENICOL	PENICILLIN†	STREPTOMYCIN
Staph. aureus									
1.	Pus	+	+	None	0.4	0.8	12.5	<0.1	25
2.	Pus	+	+	P	0.4	0.8	12.5	<0.1	25
3.	Pus	+	+	A, P, S	0.4	0.4	6.25	100	100
4.	Pus	+	+	None	0.8	0.8	12.5	<0.1	12.5
5.	Pus	–	+	None	0.8	0.8	12.5	<0.1	25
6.	Urine	–	+	None	0.8	0.8	25	100	25
7.	Eye	+	+	None	0.8	0.8	12.5	<0.1	25
8.	Urine	+	+	P	0.8	1.6	12.5	50	25
9.	Pus	+	+	P ointment	0.8	0.8	12.5	100	25
10.	Sputum	+	+	P, S	3.1	6.25	12.5	0.4	25
11.	Urine	+	–	C, P, S, T	200	25	100	25	25
12.	Urine	+	+	A, P, T	200	50	12.5	200	100
13.	Urine	–	+	A, P, S, T	200	50	12.5	200	100
14.	Pus	–	–	P, S	200	50	100	400	25
Staph albus									
1.	Conjunctiva	+	–	None	0.2	0.2	3.1	50	12.5
2.	Urine	–	–	None	0.2	0.8	6.25	3.1	6.25
3.	Urine	–	–	None	0.8	0.8	6.25	<0.1	3.1
4.	Conjunctiva	+	–	None	0.8	1.6	6.25	<0.1	1.6
5.	Conjunctiva	+	–	Unknown	3.1	3.1	6.25	<0.1	1.6
6.	Urine	+	–	C, P, S	250	25	50	50	>800
7.	Urine	+	–	A, P, S	250	25	100	1.6	>800
8.	Urine	+	–	P, T	>250	50	6.25	6.25	>800
9.	Urine	+	–	A, C, P, T	250	50	100	0.4	>800
10.	Urine	–	–	P	>250	50	12.5	100	>800

*A, aureomycin; C, chloramphenicol; P, penicillin; S, streptomycin; T, terramycin.
†Units per milliliter.

TABLE V. ENTEROCOCCUS, PNEUMOCOCCUS AND STREPTOCOCCUS

ORGANISM	SOURCE	ANTIBIOTICS* BEFORE CULTURE	SENSITIVITY, μG./ML.				
			TERRAMYCIN	AUREOMYCIN	CHLORAM-PHENICOL	PENICILLIN†	STREPTO-MYCIN
Enterococcus							
1.	Pleural fluid	P, S	0.8	1.6	12.5	0.8	25
2.	Urine	None	0.8	0.8	12.5	6.25	100
3.	Urine	P	1.6	1.6	12.5	6.25	50
4.	Urine	None	1.6	3.1	12.5	6.25	100
5.	Urine	C	1.6	3.1	25	12.5	50
6.	Urine	A, P	3.1	3.1	25	6.25	800
7.	Urine	None	6.25	3.1	25	6.25	>100
8.	Urine	None	6.25	6.25	50	12.5	400
9.	Lung	P, S	12.5	12.5	25	12.5	>800
10.	Urine	P, S, T	100	50	25	12.5	200
Pneumococcus							
1. Type III‡	Sputum	None	0.2	1.6	1.6	<0.1	25
2. Type II	Sputum	None	0.4	0.8	3.1	<0.1	25
Streptococcus							
1. Beta	Pus	None	0.2	0.4	6.25	<0.1	1.6
2. Beta	Throat	P troches	0.4	0.8	---	<0.1	12.5
3. Beta, Group B	Urine	None	0.2	0.8	3.1	<0.1	12.5
4. Beta, microaerophilic§	Vagina	None	0.2	0.2	1.6	<0.1	25
5. Gamma, microaerophilic	Pus	A, P, S	0.2	0.2	6.25	<0.1	25
6. Alpha	Stensen's duct	P	0.2	0.2	1.6	<0.1	12.5
7. Alpha§	Eye	P	0.2	0.2	3.1	<0.1	25
8. Alpha	Sputum	None	0.4	0.8	1.6	<0.1	25
9. Alpha	Sputum	None	0.4	0.4	1.6	<0.1	12.5
10. Alpha	Sputum	None	0.4	0.8	1.6	<0.1	12.5
11. Alpha	Peritoneum	None	0.8	1.6	3.1	<0.1	6.25
12. Alpha‖	Bronchial washings	P	1.6	1.6	6.25	<0.1	12.5
13. Alpha	Sputum	A, P	1.6	3.1	3.1	<0.1	6.25

*A, aureomycin; C, chloramphenicol; P, penicillin; S, streptomycin; T, terramycin.

†Units per milliliter.

‡Tested in beef infusion broth with 0.2% dextrose and 3% ascitic fluid.

§Tested in thioglycollate broth.

‖Tested in tryptose phosphate broth.

Of fifteen strains of pseudomonas resistant to more than 100 μg per milliliter of streptomycin, thirteen had been exposed to this drug in vivo, but it was not always possible to correlate resistance with previous treatment, perhaps due in some cases to inaccurate history.

The role of past therapy in producing resistance of some strains of Mimeae to streptomycin cannot be evaluated. Some of these stock strains were obtained from patients, but their past antibiotic history is unknown, as is the case with the stock Shigella strains. None of the patients with bacillary dysentery had received previous antibiotic treatment, except for penicillin in one instance.

Good correlation was noted between previous penicillin therapy and penicillin resistance among Staphylococcus strains. Thus, eight of nine *Staph. aureus* and five of seven *Staph. albus* strains requiring 0.4 or more units per milliliter of penicillin for inhibition were from patients who had had previous penicillin treatment. Three of four patients with terramycin-resistant *Staph. aureus* and two of five patients with terramycin-resistant *Staph. albus* had had terramycin treatment before culture. Some of the terramycin-resistant, coagulase-negative *Staph. albus* strains from urine were probably contaminants and may have originated from terramycin-treated patients. Several strains of staphylococci also showed a lack of correlation between treatment with aureomycin or chloramphenicol and resistance to these drugs.

DISCUSSION

Of all the organisms tested, only proteus strains were consistently resistant to terramycin, aureomycin, streptomycin, and in lesser degree, to chloramphenicol. Experimental and clinical evidence indicates that a more effective therapeutic agent is needed for proteus infections. Clinical experience with aureomycin and terramycin treatment of pseudomonas and salmonella infections has also been disappointing, although both drugs are effective against some strains of these in vitro.

The importance of strain variations in sensitivity of bacteria to antibiotics is well known. Certain groups of organisms studied herein appeared to be homogeneously sensitive, as were members of the tribe *Mimeae* to aureomycin and terramycin, or uniformly resistant, as were the proteus strains to aureomycin, terramycin, and streptomycin. Yet even among such groups, occasional strains occur which differ strikingly from the rest, such as the strain of *Proteus vulgaris* reported by Bliss and associates,[4] which was inhibited by 10 μg per milliliter of terramycin. Although all streptococci (other than enterococci) which we tested were highly sensitive to penicillin, penicillin-resistant strains of alpha hemolytic streptococcus have been recognized.[6] Similarly, while Shigella were in general quite sensitive to terramycin, two strains required much more of this antibiotic for inhibition than did the others.

Apart from these exceptional strain differences in relatively homogeneous groups, certain other organisms are notorious for wide variation in sensitivity. *A. aerogenes,* pseudomonas, enterococcus, and staphylococcus are organisms of

this type. The development of resistance is in part a quality of the bacterium; some organisms seem to develop resistant strains more readily than others. Second, certain antibiotics, like streptomycin, produce resistant variants in exposed bacteria more quickly and regularly than other drugs, although organisms like those mentioned can produce strains resistant to any antibiotic. A third important factor in the emergence of resistant strains in patients is the duration of antibiotic therapy.

Group resistance like that of proteus to aureomycin and terramycin may be considered a natural biological characteristic of the genus. The greater penicillin resistance of enterococci, as compared to other streptococci, is evidently a similar generic characteristic. In contrast, the occurrence of strains of pseudomonas that are highly resistant to aureomycin, terramycin, and streptomycin could be interpreted as resulting from the ready emergence of resistant variants in a naturally sensitive genus. The same explanation seems likely in the case of staphylococcus strains resistant to aureomycin, terramycin, and penicillin. The appearance of such resistant strains is probably not dependent upon contact with the antibiotic but is favored by it as a result of artificial selection.

It is recognized that the incidence of penicillin-resistant staphylococci encountered in hospital laboratories is increasing.[8, 16] Thus in the present study eight of fourteen strains of *Staph. aureus* collected at random from hospital patients required twenty-five or more units of penicillin per milliliter for inhibition. Four of these strains were also moderately to highly resistant to aureomycin, chloramphenicol, streptomycin, and terramycin. Since three of the four patients with terramycin-resistant *Staph. aureus* strains had been treated with terramycin before culture, while none of the ten patients with sensitive strains had received it, one is tempted to speculate as to whether terramycin resistance may develop in staphylococci after as little as two to four days of treatment. The fourth patient, however, had not received aureomycin, chloramphenicol, or terramycin; yet his staphylococcus was resistant to all.

It is of interest that no staphylococcus strains were found with intermediate sensitivity to terramycin. All were either quite sensitive or highly resistant. While many more cultures should be studied, the scant data available suggest the possibility that a sudden, huge increase in resistance to terramycin may occur, such as has been observed with streptomycin. This question lends itself to investigation in vitro and in vivo.

It has been shown that terramycin and aureomycin-resistant strains of staphylococci already occur in appreciable numbers, and such strains may be expected to increase in frequency, as penicillin-resistant strains have done, when these newer drugs are used more widely. The degree of resistance to aureomycin appeared to be somewhat less than to terramycin though presumably sufficient to interfere with therapy. While these antibiotics may be useful in treating infections with penicillin-resistant strains of staphylococcus, their value will be lessened if aureomycin and terramycin resistance also develop readily. In such cases the sulfonamides may be used.

SUMMARY

1. One hundred thirty-three strains of bacteria, most of them isolated from hospital patients, were tested in vitro for sensitivity to terramycin, aureomycin, chloramphenicol, and streptomycin. Gram-positive cocci were also tested with penicillin.

2. The antibiotic spectrum of terramycin generally resembled that of aureomycin, although terramycin inhibited pseudomonas strains in lesser concentrations and, like chloramphenicol, showed greater activity against shigella.

3. Terramycin appeared more active than aureomycin and chloramphenicol against *A. aerogenes.* Seven of ten strains were resistant to streptomycin.

4. Three *Brucella suis* strains were highly sensitive to terramycin, aureomycin, streptomycin, and chloramphenicol. When the organisms were recovered in relapse after treatment with terramycin, there was no increase in resistance to this drug.

5. Most sensitive to terramycin, aureomycin, and penicillin were pneumococcal and streptococcal strains. These required slightly higher concentrations of chloramphenicol and much higher concentrations of streptomycin for inhibition. Enterococci were somewhat less sensitive, but were inhibited by smaller amounts of terramycin, aureomycin, and penicillin than of the other antibiotics.

6. Many staphylococci were very sensitive to terramycin, aureomycin, and penicillin, but several resistant strains were encountered. Except for these, staphylococci, like streptococci, were less sensitive to chloramphenicol and least of all to streptomycin.

7. Four of fourteen strains of *Staph. aureus* and five of ten strains of *Staph. albus* were highly resistant to terramycin, requiring 200 or more μg per milliliter for inhibition. Some strains were resistant only to penicillin, others to all agents tested.

8. Striking variation was noted in sensitivity of pseudomonas and enterococcus strains to all the antibiotics.

9. Terramycin and aureomycin were highly active against ten strains of the tribe *Mimeae,* but most of these were resistant to streptomycin. Chloramphenicol was effective against two strains of *Mima polymorpha* and one of *Colloides anoxydana,* but high concentrations were required to inhibit seven strains of *Herellea vaginicola.*

10. None of ten proteus strains was effectively inhibited by the antibiotics tested, although some appeared less resistant to chloramphenicol than to the other agents.

11. Tests on three strains of *Alc. fecalis,* three of *K. pneumoniae* type B, and three salmonella strains are included.

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LABORATORY METHODS

A MECHANO-ELECTRONIC TRANSDUCER MANOMETER

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IN BIOLOGICAL pressure recording (recording of blood pressure) membrane manometers are generally used, the theoretical basis of which was founded by Frank[11] in the beginning of this century and further developed and extended by Broemser,[3-5] Wiggers,[27] Hamilton and associates[12] and Tybjaerg Hansen and Warburg,[23, 25] among others. For the transmission of the membrane movement to the recording device a fairly great number of principles have been used. In the last years, however, there has been an increasing interest in electrical transmission methods, which in many respects are preferable to mechanical or optical transmission.

Among the electrical systems used for pressure recording in the organism* we find the photoelectric,[20] piezo-electric,[15, 17] inductive,[18, 26] magneto-electric,[13] and resistance manometers. A special type of the resistance manometer is the strain gauge manometer, which was introduced to physiology four years ago[2, 14] and which also has been used in our laboratory.[7] Finally, it remains to mention the capacitance or condenser principle which has been used for instance by Lilly,[16] and in the manometer designed by Buchtal and Warburg,[6] modified by Skouby,[21] and further modified and developed by Tybjaerg Hansen and Warburg,[22, 24] so that it is one of the best clinical methods now existing.

METHODS

In the apparatus developed in our laboratory[8, 19] still another transmission is used. The manometer is based on the principle that the pressure variations influencing the membrane of the manometer are directly transmitted and transformed into electric current variations by means of an electron tube. The tube used is the RCA mechano-electronic transducer tube No. 5734. This tube which was originally designed for vibration recording is a subminiature triode. A blood pressure recording device based on the same principle has been constructed by Arnott, Cumming, and associates.[1, 9] Curtis and Nickerson[10] have used a similar transducer tube in a device for peripheral venous pulse recording.

Fig. 1 shows a cross section of the tube and its working characteristics. The staff-shaped plate is movable in relation to the indirectly heated cathode and the control grid. One end of the plate is furnished with a small shaft extending through the center of a thin metal diaphragm at the upper end of the tube. An angular displacement of the plate shaft changes the distance between the fixed grid and the plate, thus causing a change in the plate current.

Received for publication, Feb. 26, 1951.

From The Research Institute of National Defence, Department 1, Sundbyberg, Sweden.

*An extensive review of different pressure recording methods has been given by Tybjaerg Hansen.[24]

This change in the anode current can by means of a bridge coupling be used to control a recording instrument (an oscillograph) which indicates the position of the plate and the movements of the plate shaft. Only very small displacements of the plate shaft are required to give great variations in the current. Thus, for the maximum permissible angular deflection of the plate shaft, i.e., ±0.5 degrees, the change of the anode current through the 75 K ohm anode resistor gives an output voltage of about 40 volts. The length of the tube is only about 2 cm., its diameter about 8 mm., and it weighs less than 2 grams. The tube can be used without any amplification but for certain reasons, to be seen later, an amplifier ought to be used. In Fig. 2 a simplified wiring diagram is shown which we have used in some of our experiments with the pressure recorder. This type of amplifier is used, as it makes possible a static calibration of the manometer. For further details about the transducer tube see RCA Tube Hand Book.

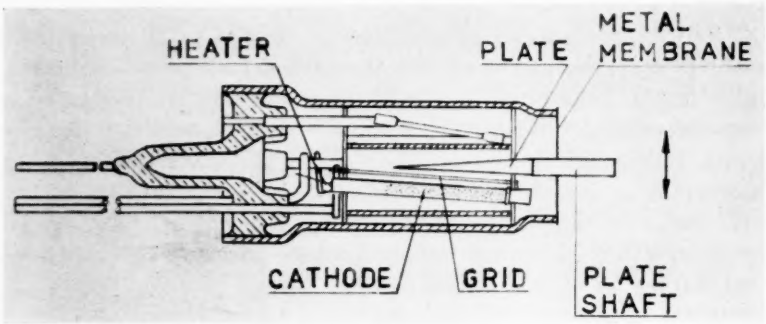

Fig. 1A.

Fig. 1A.—Cross section of the mechano-electronic transducer tube RCA 5734.

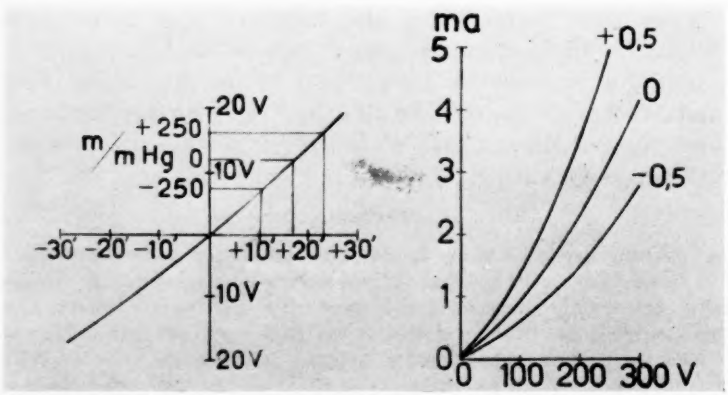

Fig. 1B.

Fig. 1B.—Left: the voltage scale is output voltage across 75,000 ohms load resistor. +250–0–-250 mm. Hg is the pressure applied to membrane (*3*). -30′ to +30′ is deflection of the movable anode. Right: plate characteristic of the tube as a function of anode (plate) displacement.

DESCRIPTION OF THE MANOMETER

The manometer (Fig. 3) consists of a conical pressure chamber (*5*) with a connection tube (*6*) with cocks (*7, 8, 9*) to the needle or catheter at the one end and the metal membrane (*3*) at the other. The housing (*10*) for the transducer tube is mounted directly to the membrane housing. The whole apparatus with the exception of the membrane is made of stainless steel.

The transducer tube is fixed in the tube housing by means of a solidifying mass thus preventing displacements of the tube. When the tube is working it gets rather hot. In order to prevent overheating the tube housing is furnished with some cooling or radiator fins. They are adjusted to give the tube and the whole apparatus a working temperature of 30 to 35° C. The plate shaft (*1*) of the tube has been perpendicularly bored through and furnished with a thin screw (*2*) which rests against the membrane. The length of the screw is 11 mm., and its diameter is only 0.6 mm.

The membrane (*3*) is made of silver or platinum, the latter metal being more suitable from some points of view (e.g., a lower coefficient of thermal expansion). The thickness of the membrane is 0.07 mm., and it has a free diameter of 10.5 millimeters.

The space behind the membrane is by means of a thin cannula (*4*) connected with the outside air in order to prevent variations of temperature in the manometer and of the surrounding air from influencing the membrane and spoiling the zero stability.

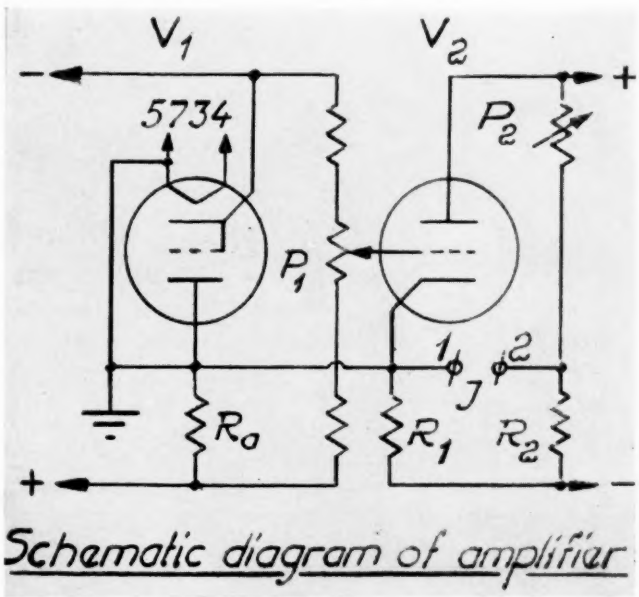

Fig. 2.—Schematic diagram of amplifier. DC plate supply of V_1 250 volts. Load resistance R_a 75,000 ohms. The other resistance values are determined by V_2 and the galvanometer.

The screw (*2*) in the plate shaft of the transducer is screwed in so much that the plate is at the central point of its positive deflection (see Fig. 1*A*, left), when no pressure is applied to the membrane (*3*). It is not necessary to have a fixed junction between the plate shaft and the membrane of the manometer, as the plate shaft, due to its tendency to return to its neutral position, is firmly pressed against the membrane (*3*). Thus, the anode of the transducer will follow the movements of the membrane in each direction, and the manometer will record negative as well as positive pressures.

The exploited part of the working scope of the tube is shown in the diagram (Fig. 1*B*, left). The reason why such a small part of the working scope of the transducer is used is that the amplification is almost linear within this range and of such a shape that it can be conveniently corrected to linearity by using a suitable amplifier. Since the transducer tube is evacuated the thin metal diaphragm through which the plate shaft extends is stretched by external atmospheric pressure, and this gives the plate a natural frequency as high as about 400 c. in spite of its mobility. The manometer can be sterilized by boiling, and in dry air it can stand heating to 150° C. When boiling the manometer a piece of rubber tubing is put on the cannula in order to prevent water from entering into it.

The best way of filling the manometer is by boiling in distilled water. However, we have found in our experiments that it can be filled in the following way without any risk of getting air bubbles in the manometer. With the cocks, (*7*) and (*8*), in open position sterile physiological saline is gently injected with a syringe through cock (*9*). The air is driven out by gently turning and shaking the manometer and by tapping on the manometer with the finger.

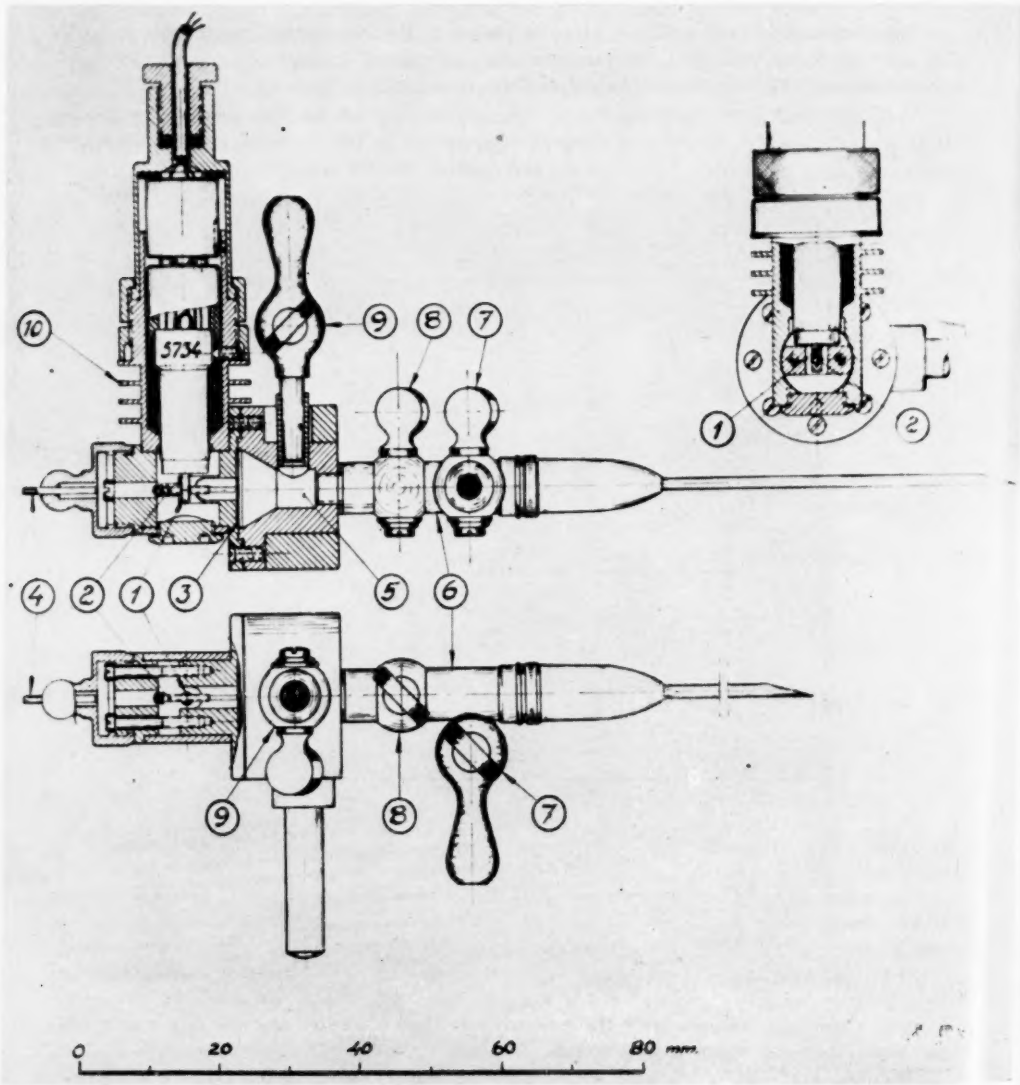

Fig. 3.—Diagram of the manometer. For explanation of figures see text.

The tube with the cock (*7*) is used for injections or for flushing the catheter. When doing this, the cock (*8*) must be closed in order to avoid a pressure rise in the pressure chamber. Checking of zero can be easily performed during a run by shutting the cock (*8*) and by opening the cock (*9*).

Physical Properties of the Manometer.—The pressure range of the manometer is ±250 mm. Hg. The capacity of the hydrodynamic system—including a needle with a length of

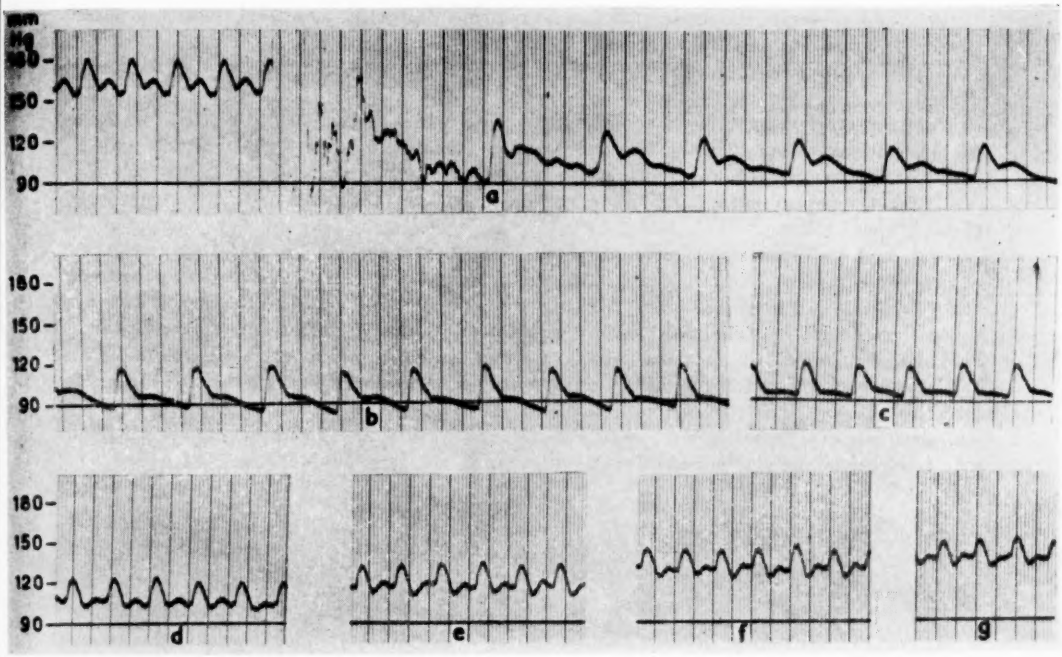

Fig. 4.—Blood pressure in the left carotid artery of a rabbit during a blast experiment. Detonation occurred at the disturbance in the upper curve. The curves *B* to *G* are obtained at different intervals during the first 4 minutes after the detonation.

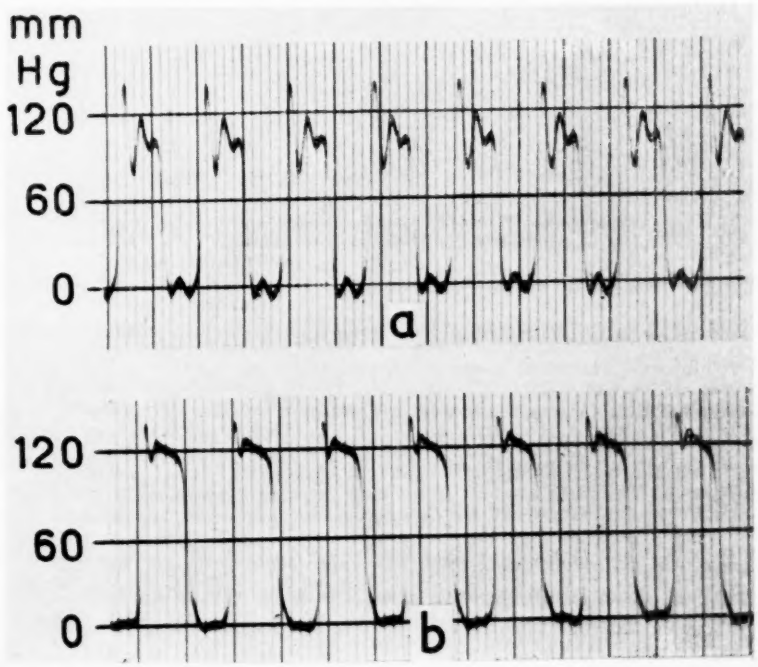

Fig. 5.—Left ventricle curves obtained by direct heart puncture of anesthetized rabbits.

70 mm. and an inner diameter of 1.8 mm. is about 1.2 cm.^{3} The total volume displacement at 250 mm. Hg amounts to about 5×10^{-5} cm.^{3}

The potential change in the test circuit is of the order of 1 V per 0.002 mm. of membrane deflection.

The calibration is stable if a constant input current is maintained. Unilateral warming of the manometer may spoil the zero stability and should be avoided. With suitable amplification, the calibration is linear within ±2 per cent.

The undamped natural frequency of the manometer including the needle mentioned above is about 150 *c*.

Figs. 4 to 6 show some curves obtained with the manometer. As recording instrument a Triplex electrocardiograph (system Elmqvist) is used. Fig. 4 shows a carotid pressure curve of a rabbit during a blast experiment. The animal was exposed to the blast wave in a detonation chamber. A shortened cardiac catheter (USCI no. 7) with a length of 20 cm. and an inner diameter of 1.2 mm. was inserted and tied in the carotid artery and connected with the manometer. Fig. 5 shows left ventricle curves from direct heart puncture of an anesthetized rabbit. The upper curve is obtained with a hypodermic needle with a length of 70 mm. and an inner diameter of 0.8 mm. The curves 5 *b* and 6 are from other rabbits obtained with a thicker needle (inner diameter 1.8 mm.).

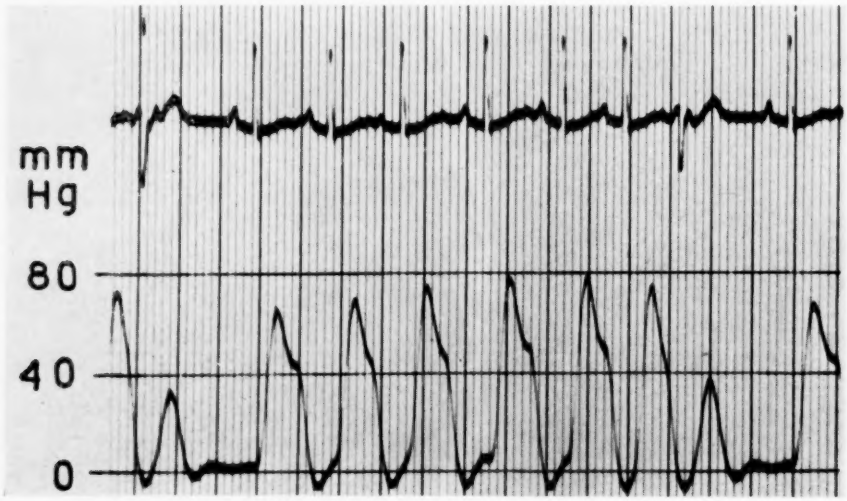

Fig. 6.—Left ventricle curve of a rabbit. Upper curve electrocardiogram.

In some experiments the manometer has been used for measurement of pressure in gas-filled cavities.

The manometer was originally designed for use in animal experiments. The apparatus will, however, be suitable also for studies of human circulatory physiology. Work is continued with some modifications of the apparatus to get suitable properties and correct damping conditions so that it can be properly used for the recording of pressure in the human cardiovascular system.

SUMMARY

A new manometer for direct pressure recording in the cardiovascular system and in other fluid- or gas-filled body cavities has been constructed. The pressure variations are directly transmitted and transformed into electric current variations by means of a mechano-electronic transducer. The construction of the manometer and its physical properties are described. Some pressure tracings obtained with the apparatus are given.

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LOCAL PROCAINE BLOCK AS AN ADDED MEASURE TO OVERCOME VENOSPASM DURING CARDIAC CATHETERIZATION

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MEASURES to prevent venospasm incident to right-sided cardiac catheterization have been described,[1, 2] but once spasm occurs catheterization must often be terminated.

METHODS

In our hands the recommended use of added sedation and local application of a heating pad or hot water bottle alone has not given satisfactory results. We have therefore resorted to local vein procainization which relieves venospasm in the majority of cases. Success of this measure depends on the usual initiation of spasm as a focal phenomenon, rather than a diffuse involvement of the entire channel from the point of entry in the anticubital fossa to the subclavian vein. If unchecked, spasm may involve the complete length of the vessel.

As spasm develops, the operator encounters increasing difficulty in moving the catheter. Simultaneously the patient often complains of pain localized over a distance of one or more centimeters, in any location between the anticubital fossa and the axilla. This area from the skin to the perivenous tissues is immediately infiltrated liberally with 2 per cent procaine. If the injection is successful, both pain and spasm will immediately subside, and motion of the catheter may be resumed.

RESULTS AND CONCLUSIONS

We have treated nine consecutive cases of venospasm in this fashion, seven of them successfully. In six cases the spasm was localized and immediately relieved. In one instance the spasm extended to the subclavian vein, where the curved tip of the catheter was seen fluoroscopically to straighten out as it was gripped by the contracting vessel. In this case procaine was injected perivenously and serially from the point of entry to the farthest point possible in the axilla compatible with safety. After a five-minute delay the catheter was moved without difficulty and the anticipated procedure was carried out to completion. This patient complained of pain along the entire course of the vessel at the onset of spasm.

In two instances, one of them representing nonresponsive diffuse spasm, the procedure failed. In the other case, that of a small recalcitrant boy, it was deemed best to discontinue attempts early.

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Received for publication March 30, 1951.

Spasm did not recur in the seven successfully treated patients following procainization. Caution must be exercised not to use excessive amounts of procaine in children, and a fifteen-minute waiting period instituted between the administration of an added half dose of a barbiturate and procainization.

The arms of all patients so treated have been inspected over a five-day period following catheterization. In no case has the procedure resulted in either infection or phlebitis.

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DETERMINATION OF POTASSIUM IN LIVER TISSUE

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RESULTS from an investigation on the volume distribution of potassium indicated that potassium must be preferentially concentrated in some of the organs of the body and that Dibenamine (N,N-Dibenzyl-β-chloroethylamine) must increase this concentration because the volume distribution is significantly increased above the controls.[1] The work of Fenn[2] in particular suggests that under the conditions of these experiments there might be a significant increase in potassium storage in the liver. The questions then arose as to whether the liver potassium concentration in the control dogs on which volume distributions were done was increased and whether the higher volume distribution that occurred with Dibenamine treated dogs was the result of a further increased liver storage.

METHODS

A simple method for determining the potassium of brain and skeletal muscle tissue without the necessity of ashing has been reported by Stone and Shapiro.[3] Their technique consisted of boiling brain and muscle tissue in 100 or 200 ml. of distilled water using a reflux condenser. After thirty-six hours the potassium content of the supernatant liquid was determined by flame photometry. To check the accuracy of their method they ashed the residual tissue at 550° C., taking it up in 0.1 N HCl and then making it up to 50 or 100 ml. with distilled water. They found no potassium in the residual tissue. No reports have appeared confirming their observations and, more important, their method of comparison did not eliminate the possibility of potassium loss during the ashing process. The tissues were ashed at a temperature of 550° C., and, at that temperature, potassium chloride has an appreciable vapor pressure. It is therefore a possibility that the small amount of potassium left in the residual tissue was lost in the ashing process. However, if the method has sufficient accuracy, the determination becomes a procedure of clinical interest.

Our procedure was to take marginal liver samples at various times during the intravenous drip of isotonic potassium chloride. The tissue was divided and one piece used for ashing and the other for boiling. At the completion of the experiment the liver was removed and a large marginal section cut. From this tissue alternate sections were selected for ashing and boiling. The tissue used for the determination ranged in weight from 0.5 to 0.75 Gm.

RESULTS AND CONCLUSIONS

Comparison of the data in Table I shows that the ashed and boiled samples gave results which are reliable to within about 4 per cent. To obtain reliable results we found it necessary to ash at 400° C., otherwise loss of potassium occurred with some samples. Also using this temperature for ashing, we found no potassium in the residual tissue.

Homogenizing of the tissue before boiling had no advantage over boiling alone as is shown by Table II. There was the added disadvantage with the

From the Department of Pharmacology, Baylor University College of Medicine.
This study was aided by grants from the American Heart Association and the Eli Lilly Company.
Received for publication, April 28, 1951.

TABLE I

SAMPLE NO.	BOILED		ASHED	
	WEIGHT*	POTASSIUM†	WEIGHT	POTASSIUM
1	0.35	1.14	0.27	1.18
2	0.32	2.49	0.36	2.50
	0.48	2.45		
	0.62	2.57		
	0.55	2.54		
	0.54	2.55		
3	0.25	1.95	0.56	1.96
			0.60	2.16
4	0.56	2.14	0.68	2.06
			0.55	2.01

*Tissue weight in grams.
†Potassium expressed as milligrams of potassium per gram of tissue.

TABLE II

SAMPLE NO.	BOILED		HOMOGENIZED	
	WEIGHT*	POTASSIUM†	WEIGHT	POTASSIUM
5	0.78	2.56	0.77	2.59
	0.63	2.46	0.52	2.50
6	0.78	2.56	0.76	2.74
	0.58	2.72	0.77	2.74
	0.63	2.46	0.76	2.70
	0.58	2.64	0.52	2.54
	0.69	2.36		
	0.59	2.42		

*Tissue weight in grams.
†Potassium expressed as milligrams of potassium per gram of tissue.

homogenized tissue in that the difficulty of transfer of the tissue was increased, and the small suspended particles clogged the atomizer of the flame photometer.

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CONTROLLED PROTHROMBIN TIME AND ANTICOAGULANT THERAPY WITH THE USE OF A SIMPLE INEXPENSIVE APPARATUS

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THE growing incidence of the older age populations is shifting the attention of the medical profession from the care of infectious diseases to research in the field of degenerative conditions. In 1948,[1] diseases of the heart and blood vessels alone took one and one-half times more lives than cancer, accidents, nephritis, pneumonia, and tuberculosis combined. In this group coronary artery occlusion and venous and arterial thromboembolic phenomena were the main problems, with coronary thrombosis accounting for nearly 200,000[1] deaths. While vascular surgery is still being employed, anticoagulants such as heparin,[2] Dicumarol, 3-3 methylenebis-(4-hydroxycoumarin),[3] tromexan,[4] danilone (phenylindanedione),[5] and Link's No. 63[6] are playing an increasing role in the prevention of embolic phenomena.

Whenever anticoagulant therapy is contemplated, measures of blood clotting such as prothrombin times must be done. Quick's[7] one-stage prothrombin time method and the simplified Simplastin[8] method are now in general use to determine the proper anticoagulant dosage. Discovery by Ware and Seeger[9] of an accelerating globulin factor and the development of a more accurate two-stage prothrombin time procedure[10] may outmode the present one-stage methods. The technical difficulties, however, in performing a two-stage test and the problem of obtaining reagents commercially render the procedure impractical for general use at the present time.

The indications for anticoagulant therapy at present consist of: (a) acute myocardial infarction, (b) acute pulmonary infarction, (c) acute venous thromboembolic disease and thrombophlebitis, (d) acute arterial embolism.

Contraindications for anticoagulant therapy include: (a) liver disease, (b) obstructive jaundice, (c) hemorrhagic diseases, (d) gastrointestinal ulcerations, (e) renal disease, (f) malignant hypertension, (g) vitamin C deficiency, (h) brain surgery, or surgery of any kind.

Clinical studies by many investigators in large medical centers[11-14] confirm the lower mortality rate among patients receiving anticoagulants for the treatment of acute myocardial infarction and thromboembolic disease. Sporadic attempts by cardiologists, internists, and surgeons are reported at maintaining anticoagulant therapy for prolonged intervals following recent myocardial infarction and thromboembolic conditions. Experiments by Blumgart[15] and his associates demonstrate that a long period of time is necessary for an adequate collateral blood supply to develop in pigs when the coronary

Received for publication, May 23, 1951.
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circulation has been altered. These findings suggest the dangers inherent in the early ambulatory stage of acute coronary occlusions. Autopsy reports of Beth Israel Hospital,[16] Boston, on patients dying of acute myocardial infarction have shown that fresh occlusions in the coronary vessels were present in two-thirds of the cases. None received anticoagulant therapy. In general, the use of anticoagulants in the treatment of coronary thrombosis and other thromboembolic disease is a worth-while procedure.

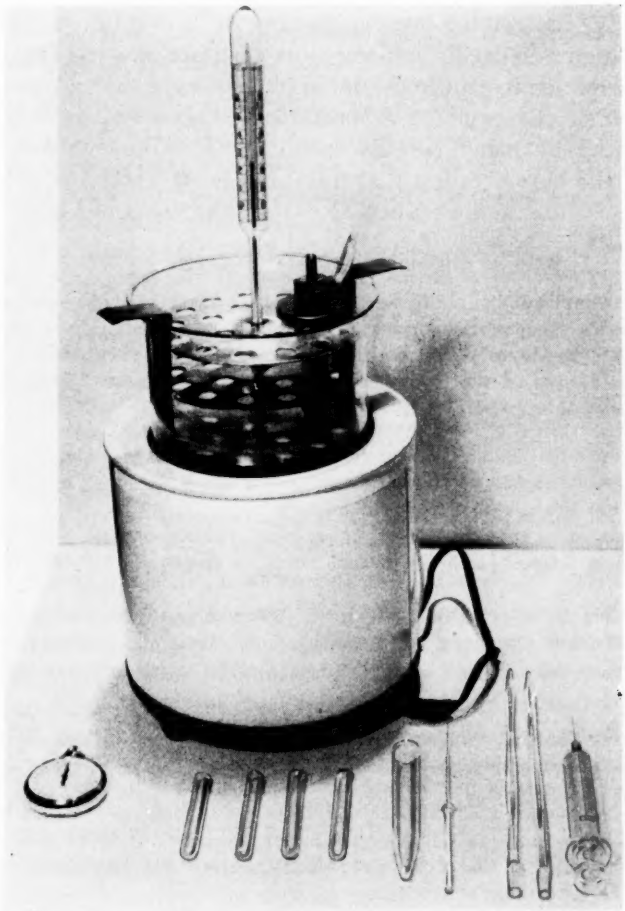

Fig. 1.

The purpose of this paper is to discuss the practical use of a prothrombin time water bath in a small office laboratory on follow-up treatment of coronary occlusions and thromboembolic conditions employing Tromexan* as an anticoagulant agent and Simplastin† as a simplified prothrombin time determina-

*Tromexan (brand of ethyl biscoumacetate) is the trademark of the Geigy Co., Inc., N. Y.

†Simplastin is the trademark of the thromboplastin prepared by the Chilcott-Maltine Laboratories, Morris Plains, N. J.

tion technique. The apparatus (see Fig. 1) was designed for office use to maintain a close clinical observation of the patient and at the same time to determine the degree of hypoprothrombinemia induced by anticoagulant therapy. Of the several anticoagulants now in use Tromexan gives promise to be least toxic for the following reasons: (1) maximum effective prothrombin time levels are usually obtained within twenty-four hours[17]; (2) it has a rapid absorption and excretion rate[18]; (3) its prolonged administration is not associated with changes in the blood picture, blod chemistry, renal or hepatic function.[19] To control Tromexan dosage the one-stage prothrombin time method, although admittedly not the most accurate, is most practical for general use in hospitals, institutions, and clinics, leaving the two-stage method to the promotion of the research laboratories. The one-stage Simplastin technique is favored by many because calcium and sodium chloride are already contained in the thromboplastin extract and is, therefore, a uniform, stable, timesaving procedure, and costs approximately ten cents per test.

DESCRIPTION OF APPARATUS

Using the Shapiro-Link machine as a control, prothrombin time studies were done on one hundred bloods to determine the accuracy of this water bath. Sera of patients under Dicumarol therapy at Memorial Hospital, Worcester, were used in this study. No significant difference in the results of the two machines was observed. In most instances the two apparatuses checked within 0.1 and 0.2 seconds.

I. *Water Bath Equipment.*—(See Fig. 1.)

1. A two and one-half quart electric beanery.*
2. A two liter Pyrex beaker set in the beanery.*
3. A circular test tube rack of stainless steel (which may be made by a tinsmith), capable of holding fifteen prothrombin time tubes, is suspended over the rim of the glass beaker.
4. A thermometer inserted in the center of the rack and resting on the same base level as the test tubes—thus assuring a corresponding temperature of thermometer and test tubes.
5. A thermoswitch† housed in a small compartment at the edge of the rack.

II. *Important Factors.*—

1. Apparatus does not require a mixing device (raising and lowering the rack a few times will serve the same purpose) as the heat is constant.
2. Two lead wires from the thermoswitch are connected to the feed wire of the beanery.
3. The beaker is so placed that the test tubes are in clear view of the technician.
4. Surface of the water is well above the top of the rack.
5. This apparatus can be used for other laboratory test requiring a water bath adjusted to 37° C.

PROCEDURE

I. *Regimen of Tromexan Therapy.*—

1. Dosage is given morning and evening.
2. Prothrombin time determinations are performed three times the first week, twice the second week, once the third week, and once weekly thereafter using an adequate control each time. The dosage of Tromexan is adjusted accordingly.
3. Screening tests as mentioned under contraindications include a nonprotein nitrogen test and bilirubin test, and, if indicated, other liver function tests.

*Manufactured by the Naxon Utilities Corporation, Chicago, Ill.
†Manufactured by Fenwal Incorporated, Ashland, Mass.

II. *General Prothrombin Time Preparation.*—

1. Scrupulously clean glassware is used.
2. Blood is withdrawn by a clean and neat venipuncture.
3. 4.5 ml. of the blood is immediately added to 0.5 ml. of a 0.1 Molar solution of sodium oxalate and is centrifuged for at least ten minutes.
4. Clear plasma is drawn off into test tube.
5. Prothrombin time of plasma is determined within four hours.

III. *Instructions for Use of Simplastin.*—

1. Exactly 4.0 ml. distilled water is added to vial of Simplastin and evenly mixed (approximately three minutes).
2. 0.2 ml. of Simplastin suspension is pipetted into a small serological test tube placed in a water bath at 37° C. for three minutes.
3. 0.1 ml. of plasma (previously adjusted to 37° C.) is transferred to the tube containing 0.2 ml. Simplastin suspension. Start stopwatch simultaneously.
4. Wire loop is immediately inserted and moved across tube in sweeping motion until clot appears. Watch is stopped and time recorded.

In using office water bath (Fig. 1) one hour should be allowed for the temperature to reach 37° C. For the doctor's office not equipped with a centrifuge, plasma for the prothrombin time may be obtained by allowing the oxalated blood to set for one hour or more. In the one hundred tests (previously mentioned) that were run, centrifuged and noncentrifuged samples of the same oxalated blood were checked for prothrombin time results. No difference was noted in the prothrombin time determination of the specimen that was spun down and the one that was allowed to set. It is believed that these findings are constant for tests run in a room of normal temperature.

RESULTS

Fifteen patients are now under observation in this laboratory. The plan is to determine: (1) the long range effect of Tromexan therapy upon the working capacity of individuals with a past coronary occlusion; (2) the incidence of complications from Tromexan overdosage; and (3) the likelihood of additional thromboses from underdosage. Using the small office water bath (Fig. 1), prothrombin time studies were made on each patient with the object of maintaining a hypoprothrombinemia of twenty-five to thirty seconds. Three hundred milligrams of Tromexan were given at 7 A.M. and 7 P.M. for at least two to three days. At each visit the patient was interviewed and examined while the prothrombin time was being performed by the technician. In this manner the subsequent dosage of Tromexan was determined before the patient left the office. Meanwhile, to check against bleeding a microscopic examination of the urine was done at each visit.

In this study no untoward effects have been noted in any patient from the use of Tromexan. Eight of the fifteen patients previously mentioned have been followed for a period of three months. Two are engaged in moderately vigorous work, ten to twelve months after a coronary occlusion. Prior to the use of the anticoagulant, Tromexan, both had frequent attacks of angina pectoris requiring nitroglycerin for relief; subsequently no further attacks have occurred. Except for mild skin rash in one instance, no complications have been encountered from Tromexan therapy. In one instance the prothrombin time reached fifty seconds but promptly dropped to twenty-five seconds within a twenty-four hour period following cessation of medication, confirming the rapid excretion rate and relative safety of Tromexan as a

therapeutic agent. Another patient admitted to the hospital with acute myocardial infarction developed a second coronary occlusion while hospitalized. Unfortunately, no anticoagulants were given previous to his second infarction; however, with the use of Tromexan he made an uneventful recovery. It was consistently noted in this series that a smaller dosage of Tromexan was required to produce the desired hypoprothrombinemia in the moderately active individual than in the bedridden.

DISCUSSION

Anticoagulant therapy now in use in many clinics[11-14] may have some effect in reducing the high death rate[1] from myocardial infarction and thromboembolic disease. Many investigators are using anticoagulants prophylactically on occasions, especially if repeated coronary and pulmonary incidents have justified such measures.

Although admittedly not the most accurate, the simplicity of the one-stage prothrombin time method using the Simplastin technique in ascertaining the proper dosage of Tromexan therapy should invite the attention of the medical profession to its importance. The lifesaving value of anticoagulants in acute emergencies is becoming more obvious. The procedure appears practical and sound, and should not be replaced until a more constructive, rational medical program can be developed or until new research in the etiology, histogenesis, and management of degenerative disease will make obsolete its present usage.

SUMMARY

Clinics employing anticoagulant therapy have generally received gratifying results. A sharp reduction in mortality rates from emboli and occlusion has been reported.

The indications and contraindications for anticoagulants have been outlined.

A simple inexpensive apparatus for office prothrombin time determinations is discussed.

The technique of its employment is presented.

Tromexan therapy seems safe as an office procedure; no untoward effects from its use have been observed.

Acknowledgment is made to the Memorial Hospital for the cooperation of its Laboratory in this study.

Tromexan and Simplastin were furnished by the Geigy Company and the Chilcott Laboratories, respectively, for these studies.

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A MODIFICATION OF THE ANTISTREPTOLYSIN TEST

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SINCE Todd[1] demonstrated the antigencity of streptolysin O, tests have been devised to estimate antistreptolysin O by Hodge and Swift,[2] Coburn and Pauli,[3] Massell and Miller,[4] Ipsen,[5] and Rantz and Randall.[6] With these methods several dilutions of a sample of serum have to be made, varying amounts of one dilution have to be measured, and, in the first three, a preliminary and a final titration has to be done. Sometimes, measurements of 0.05 or 0.005 ml. are necessary. These are far too cumbersome for a routine procedure in an ordinary clinical laboratory if large numbers of sera are to be examined. Furthermore, with four of the five suggested methods, the end point is the tube in which *no trace* of hemolysis occurs. As is known, an absence of hemolysis is rather difficult to determine with certainty. This can be explained by the fact that, when percentages of hemolysis are plotted against either the concentrations of complement[7] or of streptolysin "O,"[8, 9] a sigmoid curve is formed. Thus at both ends of the curve, the increments of the amount of hemolysis are small for rather large increases of the concentrations of the reagents. Therefore, the rather inaccurate end point does not warrant a closely placed series of dilutions of sera as recommended.

On account of these facts, it seemed desirable to develop a modified technique that would satisfy the following requirements, viz: (1) it should be simple enough to be used routinely in a serological laboratory, (2) it should be reasonably accurate, and (3) its ASLO* unit should be related to the previous unit in a manner so as to permit easy comparison. It is the purpose of this paper to report certain investigations made to achieve the foregoing aims.

MATERIALS AND METHODS

(a) *Streptolysin "O" (SLO).*—

A strain† of Lancefield group A, type 3 hemolytic streptococci (D58) was used for producing SLO according to Hodge and Swift's[2] or Massell and Miller's[4] technique. Five lots were made at different times during the last half year with Todd and Hewitt's broth or Hodge and Swift's broth. Reduction was accomplished by the addition of NaOH and sodium hydrosulfite (1 Gm. per liter) or cysteine HCl alone (1.5 mg. per milliliter). It was found that the hemolytic activity of several lots of SLO broth increased rapidly with the increasing amounts of cysteine added until 1.5 mg. per milliliter was reached. Larger

From Section of Preventive Medicine, Yale University School of Medicine.
Aided by a grant from Life Insurance Medical Research Fund.
Received for publication, May 28, 1951.
*ASLO = antistreptolysin "O."
†Generously given by Mrs. Rebecca C. Lancefield of the Rockefeller Institute for Medical Research, New York, N. Y.

quantities (such as 2.0 and 2.5 mg. per milliliter) of the reducing agent did not enhance the activity of the preparations to any appreciably greater degree. When cysteine is used, the container of SLO need not be sealed with petrolatum or wax. It is added directly to the SLO broth at least ten minutes, but not longer than thirty minutes, before the reduced SLO is diluted and used for the test.

(b) *Red-blood-cell Suspension* (RBC).—

Rabbit blood was obtained, defibrinated, and washed until free from hemolysis. The washed blood cells were centrifuged until no further changes of the volume of the packed cells were noted. A 5 per cent suspension was made with buffered saline (vide infra). The amount of hemoglobin therein was approximately 9 Gm. per 100 ml. In each experiment, the suspension of red cells was adjusted to contain this amount of hemoglobin.

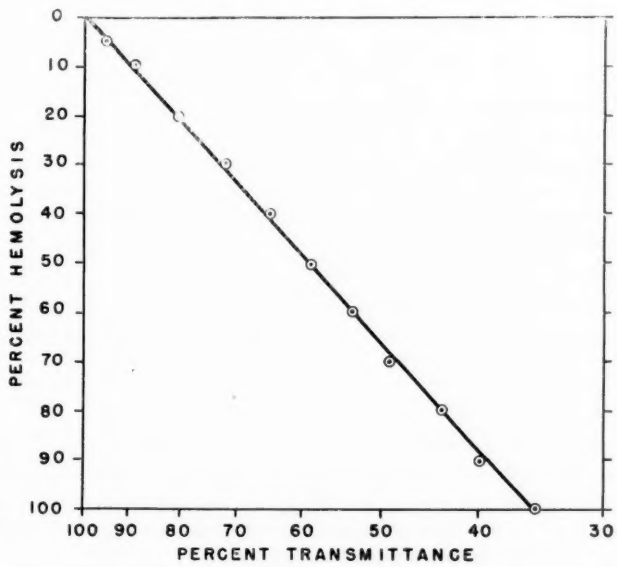

Fig. 1.—Spectrophotometric readings for percentage hemolysis.

Previous investigators advised the use of *fresh* rabbit blood. We found that this is inconvenient when a daily determination of large numbers of sera is the routine. Furthermore, the fragility of red blood cells seems to vary with individual rabbits. To avoid such errors, sufficient amounts of blood from several rabbits are pooled and preserved in Alsever's solution.* The mixture is stored in a refrigerator for at least three days before it is washed five times with large quantities of buffered saline and a 5 per cent suspension of red blood cells is prepared. For a day's use, sufficient amount of the mixture was taken out with a sterile pipette with strict aseptic precautions.

(c) *Standard sera (ASLO).*—Two sera were used at the same time for each single experiment. By Hodge and Swift's method one was estimated to contain 200 units of antistreptolysin "O" and the other 2,000 units. They were heated at 56° C. for thirty minutes immediately before use.

*Alsever's solution contains dextrose 2.05 Gm., sodium chloride 0.42 Gm., sodium citrate 0.8 Gm., and citric acid 0.055 Gm. per 100 ml. distilled water. The solution is sterilized by autoclaving at 15 pounds for eight minutes. One volume of blood is delivered into one volume of the solution. The mixture is then gently shaken.

(d) *Buffered saline (B/S).*—A mixture of one part of 0.158M phosphate buffer at pH 6.5, and one part of 0.9 per cent NaCl was found by Herbert and Todd[10] to be optimal for the SLO reaction. This B/S was used in the preparation of the suspension of red cells, for the dilution of serum, and to make streptolysin solutions.

(e) *Estimation of percentage of hemolysis.*—The 5 per cent red cell suspension as described above was diluted 1:4 with distilled water to conform with the final concentration in each test tube. This was taken as 100 per cent hemolysis. From this stock solution of hemoglobin, solutions of 90, 80, 70, 60, 50, 40, 30, 20, 10, and 5 per cent were made. A further 1:3 dilution was made with each concentration as in the actual test before readings were taken with a spectrophotometer. A graph was made with the averages for each concentration from determinations of three different preparations (Fig. 1). Reference was always made to this graph to estimate the amount of hemolysis.

(f) *Spectrophotometer.*—A Coleman, Jr. model 6A was used with a wave-length of 520 mμ, and cuvettes of 10 by 75 mm. dimension were employed.

(g) *Tests for Hemolysis.*—To 1.0 ml. of buffered saline (B/S) alone or to 1.0 ml. B/S containing one unit of antistreptolysin (as determined by Hodge and Swift's method[2]) a volume of 0.5 ml. B/S containing various amounts of streptolysin "O" was added. The tubes were shaken and incubated in a water bath at 37° C. for fifteen minutes. Then 0.5 ml. of a 5 per cent suspension of red cells (RBC) was added into each tube. The tubes were shaken and incubated at 37° C. for fifteen minutes. They were shaken again, and then incubation was continued for another thirty minutes, after which the tubes were centrifuged. It should be noted that the total volume in each test tube was 2 ml., and the final concentration of RBC was 1.25 per cent.

One milliliter of the supernatant fluid from each tube was diluted 1:3 by the addition of 2.0 ml. of distilled water to facilitate the estimation of the amount of hemolysis with the aid of a spectrophotometer. When the supernatant was pipetted out for making further dilution, care was taken not to disturb the packed red cells at the bottom of each tube.

(h) *Antistreptolysin Test.*—Twofold serial diutions of serum was used, starting with 1:50 up to 1:12,800. The volume of diluted serum was 1.0 ml. in each tube. A fixed amount of SLO (in 0.5 ml. B/S) was added into each tube. Otherwise, the procedure was exactly as described above in (g). All the sera used in this investigation were heated at 56° C. for thirty minutes before a titration was done.

(i) *Estimation of the 50 per cent end point.*—Though a spectrophotometer was used in the present investigation to ascertain the 50 per cent hemolytic end point, for visual comparison it was found reasonably accurate to employ a series of standards with various amounts of hemolysis from 10 to 100 per cent,* with 10 per cent intervals between two consecutive tubes. At the end of a titration, the tubes with definite hemolysis were compared with these standards. If one of them showed 50 per cent hemolysis, it was the end point. If, for example, in one tube there was 10 per cent hemolysis and in the next 70 per cent, the 50 per cent end point must be somewhere between the two. This point can be obtained graphically. In order to do so, the percentages of hemolysis are converted into probits[11] and the sigmoid hemolysis curves are transformed into straight lines. Therefore, a nomogram can be constructed (Fig. 2). The two sides of the graph represent the two tubes with hemolysis less than and over 50 per cent, respectively [designated (r) and (r + 1) in the graph]. The interval between them is equivalent to log 2, because the difference between the dilutions of serum in two consecutive tubes is a factor of 2. Lines are drawn to connect the various percentages of hemolysis on two sides of graph. They intersect the horizontal line connecting the 50 per cent points. The intersecting points are, therefore, the 50 per cent end points. In our example, the line connecting the 10

*The 100 per cent hemolysis here is equivalent to the final concentration of RBC in each test tube, i.e., 1.25 per cent of the packed RBC [see (d)].

and 70 per cent points intersect the 50 per cent line at 0.70. That means that the 50 per cent end point of our example is located at a point 7/10 of the total distance between (r) and (r + 1), from (r). The actual dilution of serum at this end point equals: (dilution

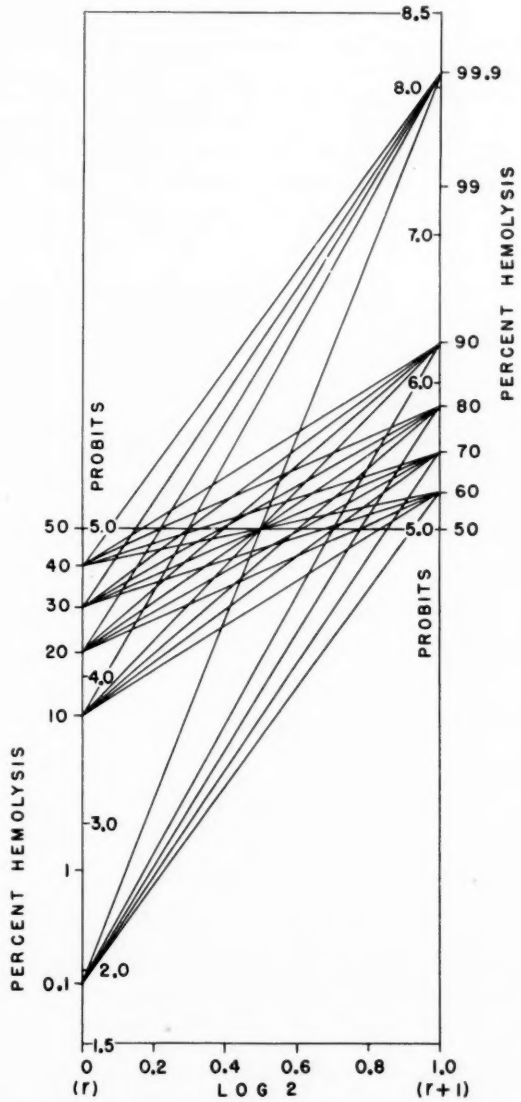

Fig. 2.—Nomogram for interpolating 50 per cent end points. (r) = the tube with less than 50 per cent hemolysis; $(r+1)$ = its immediate next tube with hemolysis over 50 per cent.

of serum in rth tube) $\times 2^{0.70}$. Suppose the dilution in the tube with 10 per cent hemolysis is 1:400 and that with 70 per cent is 1:800. The 50 per cent end point of our example should be $400 \times 2^{0.70} = 400 \times 1.62 = 648$ units, or 1:648.

To facilitate the calculation of the 50 per cent end points, Table I is constructed, listing various percentages of hemolysis in the two consecutive tubes (r) and (r + 1).

The numbers at the intersections of a pair of percentages are the conversion factors. With the above example of 10 and 70 per cent hemolysis in two consecutive tubes, 1.62 is the conversion factor. For instance, if the percentages are 30 and 80, the conversion factor is then 1.30. Suppose the dilution of the serum in the tube with 30 per cent hemolysis is again 1:400, the actual ASLO titer (or the 50 per cent end point) should be $400 \times 1.30 = 520$ units or 1:520. The numbers in parentheses immediately following the conversion factors in Table I are the corresponding exponents of 2. (0.70) and (0.38) are the exponents for the two examples, respectively. They are used for two purposes: (1) for the localization of the ASLO titers on a graph. With the two above examples, the end points are located 70/100 and 38/100 respectively of the interval between 1:400 and 1:800, and from the point of 1:400; (2) for coding the ASLO titers (see next section). The corresponding codes for the above examples are 4.70 and 4.38.

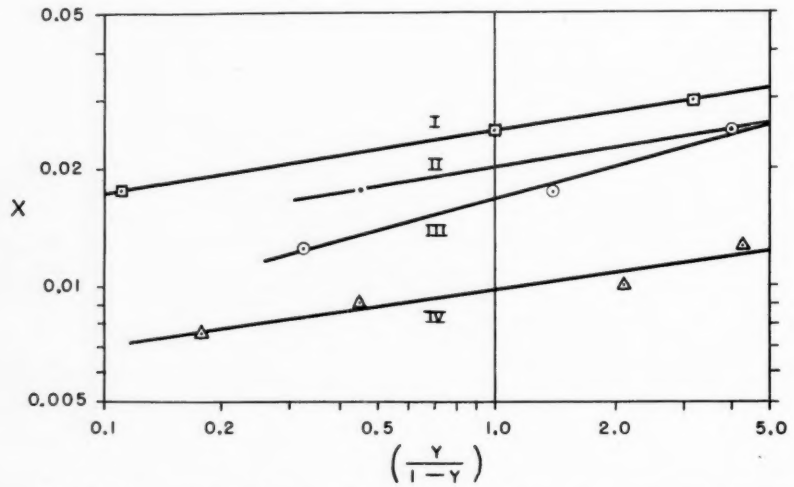

Fig. 3.—Estimation of HU50. X = amounts of SLO; Y = percentage hemolysis.

It should be noted that in Table I, 0.1 per cent and 99.9 per cent of hemolysis are listed instead of 0 and 100 per cent. This is because probits of 0 and 100 per cent are not available, and 0.1 per cent is no different from 0 per cent, nor 99.9 per cent from 100 per cent when the amount of hemolysis in test tubes is read with the naked eye. Therefore 0.1 per cent and 99.9 per cent in the table should be used as 0 per cent and 100 per cent, respectively.

(j) *The calculation of mean titers by coding.*—The mean titer of a collection of sera may be required as in serologico-epidemological studies or in estimating experimental error. With this series of twofold dilutions of serum, the reciprocal of the dilution in the first tube is ½ (initial dilution) $\times 2^1$, and the second tube, ½ (initial dilution) $\times 2^2$, . . . , and in the nth tube, ½ (initial dilution) $\times 2^n$. Thus, the tubes may be coded with exponents of 2, ____ that is, the first tube as 1, the second as 2, . . . and the nth as n. With this code the mean titer of a collection of sera may be calculated as usual. If the mean, in terms of the code, is $\times$, the titer should be ½ (initial dilution) $\times 2^{\times}$ units. The initial dilution of serum is that in the first tube of the series (in our case, 1:50). Therefore, a titer of 648 (as the example in the above section) should be ½ (50) $\times 2^{4.70}$, or 4.70 in terms of the code.

RESULTS

(1) *Estimation of the Hemolytic Activity of Streptolysin O.*—In these estimations, each tube contained the following reagents: *SLO* (in 0.5 ml. *B/S*), *B/S* (1.0 ml.) and 5 per cent *RBC* (0.5 ml.).

The amounts of SLO varied from 0.01 to 0.3 ml.* Fig. 3 shows the results of one such experiment. In this and the following, von Krogh's equation,[12] $X = K\left(\frac{Y}{1-Y}\right)^{\frac{1}{n}}$, was applied to estimate the 50 per cent end point. Here X = the amount of streptolysin. Y = the percentage of hemolysis. $\frac{1}{n}$ = the slope of the line when log X is plotted against $\log\left(\frac{Y}{1-Y}\right)$. K = a constant and equals X at the 50 per cent end point. The 50 per cent end points of a hemolytic unit (or *HU 50*) of four lots of SLO were 0.025 ml., 0.020 ml., 0.0097 ml., and 0.017 ml., or 40, 50, 160, and 50 units per ml., respectively (Fig. 3 and Table II).

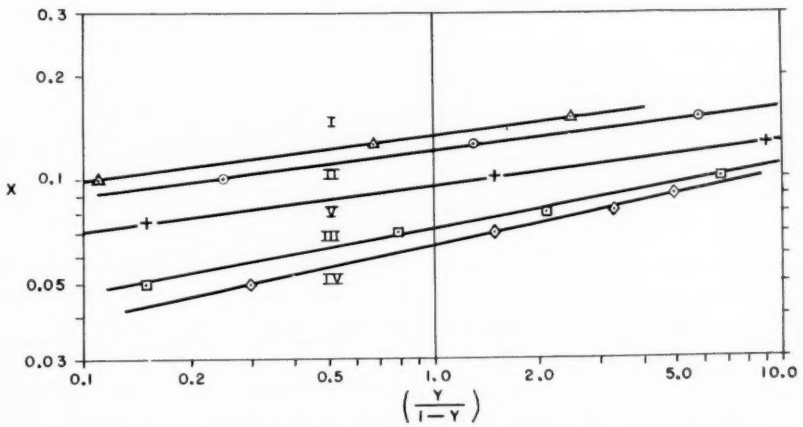

Fig. 4.—Estimation of ASC50. *X* = amount of SLO; *Y* = percentage hemolysis.

TABLE I. CONVERSION FACTORS FOR 50 PER CENT END POINTS

(r + 1) / (r)	60%	70%	80%	90%	99.9%
0.1%	1.89 (0.92)	1.81 (0.86)	1.71 (0.78)	1.62 (0.70)	1.41 (0.50)
10%	1.79 (0.84)	1.62 (0.70)	1.51 (0.60)	1.41 (0.50)	1.23 (0.30)
20%	1.71 (0.78)	1.53 (0.62)	1.41 (0.50)	1.32 (0.40)	1.16 (0.22)
30%	1.60 (0.68)	1.41 (0.50)	1.30 (0.38)	1.23 (0.30)	1.10 (0.14)
40%	1.41 (0.50)	1.25 (0.32)	1.16 (0.22)	1.12 (0.16)	1.06 (0.08)

(r) = the tube with hemolysis less than 50 per cent.
(r + 1) = the tube with hemolysis over 50 per cent.

TABLE II. AMOUNT OF SLO IN DIFFERENT PREPARATIONS AS DETERMINED BY THE TWO METHODS

	HU50				ASC50				
NO. OF PREPARATION	I	II	III	IV	I	II	III	IV	V
Units per milliliter	40	50	106	50	7.7	8.3	14	16	11

*In order to eliminate the error arising from measuring small amounts, a stock solution of SLO (usually 20 to 30 ml. or more) was made containing 0.3 ml. SLO in 0.5 ml. B/S. From it, dilute solutions were made to contain the required amounts of SLO.

(2) *The Estimation of Antistreptolysin-Combining (ASC) Capacity of SLO.*—In these experiments, each tube contained the following reagents: *SLO* (in 0.5 ml. B/S), 1 unit *ASLO* (in 1 ml. B/S) and 5 per cent *RBC* (0.5 ml.). The amounts* of SLO used in this test were 0.150, 0.125, 0.100, 0.075, and 0.050 milliliter. Fig. 4 and Table II show the amounts of the five preparations of SLO required to give rise to 50 per cent hemolysis (0.13 ml., 0.12 ml., 0.071 ml., 0.064 ml., and 0.095 ml., or 7.7, and 8.3, 14, 16, and 11 units per milliliter, respectively). This end point may be called *ASC50.*

(3) *Antistreptolysin O Test Using HU50 as a Unit of SLO.*—One *HU50* of each of the four preparations of SLO was added to a series of twofold dilutions of the standard sera.

The results are shown in Table III, with respect to the two standard sera. The 50 per cent end points for No. 2 serum varied between 2,300 and 10,500, and those for No. 1 serum between 153 and 334.

TABLE III. AMOUNT OF ASLO IN TWO STANDARD SERA AS ESTIMATED IN TERMS OF HU50 AND ASC50

NO. OF PREPARATION OF SLO	HU50				ASC50				
	I	II	III	IV	I	II	III	IV	V
Standard Sera									
1	163	153	334	280	200	200	200	200	200
2	2800	2300	10500	3200	2100	2100	2100	2100	2100

(4) *ASLO Test Using ASC50 as a Unit of SLO.*—When *ASC50* of the five lots of SLO were taken as a unit, the 50 per cent end points for No. 1 serum were 200 and those for No. 2 serum 2,100 units (Table III).

(5) *Experimental Error.*—Attempts were made to estimate the error inherent in this technique. One serum was examined eleven times with different pipettes and glassware each time. The results are shown in Table IV, columns 1 and 2. For this particular serum, the mean ASLO titer was 668 units (or $\frac{50}{2} \times 2^{4.75 \pm 0.02}$, or ranging from 664 to 684 units). From this, we can see that the experimental error was very small, far less than a twofold difference.

TABLE IV. EXPERIMENTAL ERROR

% HEMOLYSIS IN		CODE*	ASLO*
4TH TUBE (1)	5TH TUBE (2)	(3)	(4)
0	80	4.78	684
0	80	4.78	684
0	80	4.78	684
0	80	4.78	684
0	80	4.78	684
0	85	4.74	668
0	85	4.74	668
0	85	4.74	668
0	85	4.74	668
0	90	4.70	648
0	90	4.70	648
	Mean	4.75	668
	Standard error	±0.009	

Fiducial limits = $\pm 0.009 \times 1.96 = \pm 0.0176$, when P = 0.95. Therefore, the true ASLO titer was between $\frac{1}{2} \times 50 \times 2^{4.73}$ and $\frac{1}{2} \times 50 \times 2^{4.77}$, or between 664 and 684 units.

*See sections (i) and (j), and Table I.

Twenty sera with different ASLO titers were selected at random. Most of them were stored at –70° C. for at least one month. They were titrated on three occasions, each repeated by two persons so as to ascertain the possible magnitude of error due to individual differences. On the same serum, a difference in results may or may not be demonstrated with the two individuals on the same day or on different days. Thus, eighty-six such differences were accumulated. The absolute values of the differences were used for the calculation of the possible amount of error. The mean difference was $2^{0.23}$, with a fiducial limit of $2^{\pm 0.049}$. If we take $2^{\frac{\pm 0.23 \pm 0.05}{2}}$ or $2^{\pm 0.14}$ as our experimental error, due to individual differences, the probability of having an ASLO titer within these limits is about 95 per cent. This is again far smaller than a twofold difference of ASLO titer. For example, for a titer of 668 units, the true value should range from 612 to 740 units (or $\frac{50}{2} \times 2^{4.75 \pm 0.14}$), with an error of approximately ±64 units. It should be noted that the amount of error varies with the magnitude of the estimated titer and is usually about ±10 per cent of the titer. The following table (Table V) serves as a guide to show the amount of error at the various levels of ASLO titers, the center points of which were used as the imaginary ASLO titers.

TABLE V. THE MAGNITUDE OF ERROR AT VARIOUS RANGES OF ASLO TITER

LEVEL OF ASLO TITER (UNITS) $\left(\frac{50}{2} \times 2^{r} \text{ TO } \frac{50}{2} \times 2^{r+1}\right)$ (1)	TITER (UNITS) AT CENTER OF LEVELS IN (1) $\left(\frac{50}{2} \times 2^{r+0.5}\right)$ (2)	POSSIBLE RANGE OF THE TITER IN (2) $\left(\frac{50}{2} \times 2^{r \pm 0.64*}\right)$ (3)	APPROXIMATE MAGNITUDE OF ERROR (4)
50- 100	71	64- 78	±7
100- 200	141	128- 156	±14
200- 400	283	256- 312	±28
400- 800	565	512- 624	±56
800-1600	1130	1024-1248	±112
1600-3200	2260	2048-2496	±224

*0.64 = 0.50 + 0.14, here 0.14 is the estimated experimental error.

DISCUSSION

To estimate the hemolytic unit of SLO it seemed obvious to make a direct determination of its hemolytic capacity. Nevertheless, one such unit (i.e., HU50) of SLO in different batches gave different 50 per cent end points with the same serum. However, when the unit of SLO was defined in terms of its antistreptolysin-combining capacity, the results were the same for the same serum with different preparations of the lysin. This can be explained by the fact that ASLO combines with SLO in its oxidized form as well as in its reduced form. Different lots differ not only in their total amounts of SLO but also in the proportion of SLO reduced to the oxidized form. Thus, batches III and IV had almost the same combining capacity, but III had more hemolytic activity than IV (see Figs. 3 and 4, and Table II). In the ASLO test, the hemolytic activity as well as the combining power of SLO is involved. This confirmed the observation made by Hodge & Swift.[2]

With Hodge and Swift's,[2] Coburn and Pauli's,[3] Massell and Miller's,[4] and Rantz and Randall's[6] methods, the end point was taken as the tube with *no trace* of hemolysis. This point is difficult to determine accurately with the naked eye. However, at the region of 50 per cent hemolysis, a small increase of SLO will lead to a great increase of hemolysis. Thus, the 50 per cent end point can be determined accurately. It is obvious that the 50 per cent hemolysis point should be used as the end point. Ipsen's[5] method of comparing the 50 per cent hemolytic end point with a standard serum was a definite improvement. However, his method is too elaborate to be practical, and also required differences in measurements of as little as 0.05 ml., when the total volume of the reagents contained in each tube ranged from 2.05 to 2.20 milliliters. It has been demonstrated that hemolytic activity of a reagent varies with the total volume of the lytic system.[13] Thus, it seems advisable to retain the original proportions of the reagents and the total volume (2 ml.) recommended in Hodge and Swift's method.[2]

The optimal concentration of red blood cells for this test was investigated. It was found that when a small amount of SLO was added to various concentrations of RBC, the amount of hemolysis varied inversely with the amount of red cells. There was more hemolysis in tubes with less red cells than in those with more cells (Fig. 5). This confirmed the observation of Herbert and Todd[10] which was made in a different manner. It is of interest to note that Rantz and Randall[6] used different amounts of a 5 per cent red cell suspension, 0.5 ml. for determining the combining unit of SLO and 0.15 ml. for ASLO titer. Insofar as the underlying policy of the present investigation was to make the least amount of modification when necessary and to interpret the future results thus obtained in terms of the previously described techniques, and the 5 per cent suspension of RBC has been used with satisfaction for such a long time; there seemed no need to make an alteration in this respect, particularly because hemolytic capacity of SLO was a function of the concentration of red cells.

By the present technique a unit of SLO was defined as the amount of SLO which, after mixing with one unit ASLO, gives rise to 50 per cent hemolysis. The original unit of ASLO in our standard sera was estimated by Hodge and Swift's method.[2] One of them varied from 100 units to 300 units, but for about two-thirds of the times, it was 200 units. The other was estimated with the same technique to contain 2,000 units per milliliter. Because the Hodge and Swift technic was always used as our reference to determine ASLO unit which was in turn used to define SLO unit, the results are more or less comparable.

As the dilutions of serum were concerned, Hodge and Swift's[2] series was 1:25, 1:50, 1:100, 1:150, 1:200, 1:250, 1:300, 1:350, 1:400, 1:450, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000, 1:1200, 1:1400, 1:1600, 1:1800, and 1:2000; Massell and Miller's[4] had a difference of log 0.1 between two consecutive tubes; Loefgren[14] had a difference of log 0.125 between two tubes; Rantz and Randall's[6] series had the following unit in each tube, viz. 12, 50, 100, 125, 166, 250, 333, 500, 625, 833, 1250, and 2500; and Ipsen[5] had a series of twofold dilutions. The

series with twofold dilutions of serum was used in this investigation, because it could be done easily and appeared to minimize the errors due to variation in the calibration of pipettes used in an ordinary laboratory, in the technics of pipetting, and in other manipulations, as indicated by a small experimental error.

It is realized that the small error might be due to the fairly widely spaced twofold dilutions of serum employed in this technic. Furthermore, if one tube shows no hemolysis and its next tube shows 100 per cent hemolysis, it does not necessarily indicate that the amount of serum in the first tube just neutralizes the amount of SLO, and the excess of SLO in its next tube is just enough to cause complete hemolysis. It is quite possible that the first tube may contain an excess of ASLO, and in its next tube the amount of SLO may be far more

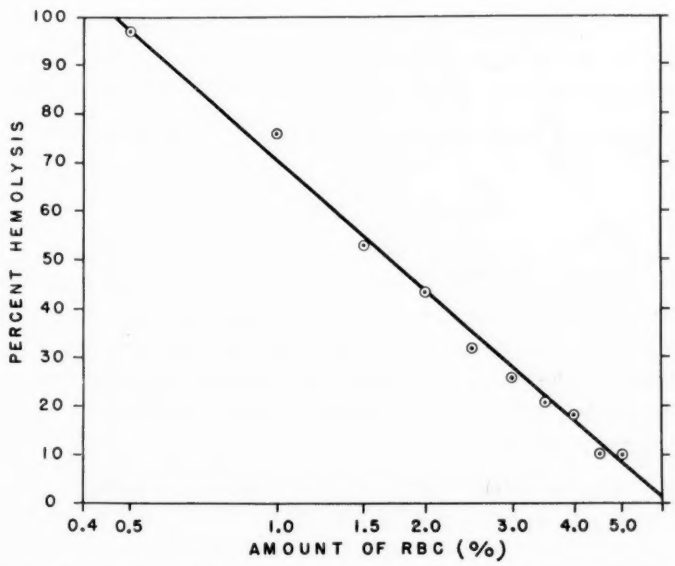

Fig. 5.—Percentage of hemolysis as a function of the concentration of RBC.

than needed for 100 per cent hemolysis. Any amount of ASLO, if greater than that needed to neutralize a certain amount of SLO, will be manifested as an absence of hemolysis. The same is true with an excess of the lysin, greater than that needed for a complete hemolysis, there shall be no more than 100 per cent hemolysis. Therefore, experiments were conducted for more precise determinations of the 50 per cent end point with dilutions of serum more closely spaced than a twofold difference, viz. 1-½ fold or 1-⅓ fold (that is, two volumes of diluted serum being added to one volume of B/S, or three volumes of diluted serum to one volume of B/S respectively when dilutions were made). Repeated titrations were made on ten sera, which had no hemolysis in one tube and 80 to 100 per cent hemolysis in the immediate next tube according to the technique with twofold dilutions. Practically all of the results fell within the estimated

limits of the experimental error. It seemed, therefore, quite unnecessary to space the difference between dilutions of serum smaller than a twofold, particularly if the limitations of such a serological test are fully realized.

It cannot be overemphasized that errors due to measurements of reagents should be minimized as much as possible by proper precautions. Thus, endeavors to measure volumes of fluids less than 0.1 ml. should be discouraged in a clinical laboratory. When the initial dilution of serum is made, the pipette employed to measure the amount of an undiluted serum should be discarded after the delivery of the required amount of serum and a clean pipette is used to transfer the diluted serum and to make the serial dilutions. From our experience, this could help to minimize the magnitude of errors. During a titration of ASLO, the amount of SLO must be so adjusted that the result of the standard ASLO falls within the above indicated limits of experimental error.

In view of the presented evidence, it may be suggested that a twofold difference of ASLO titers could be considered really significant. Here, a twofold difference means an error of $2^{\pm 1/2}$. In other words, the limits shall be ± 0.5. Our estimated standard error was 0.025. Thus $t = \frac{0.5}{0.025} = 20$.

SUMMARY AND CONCLUSION

For standardization of streptolysin O (SLO) and for estimation of antistreptolysin O (ASLO) titers, modified methods suitable for a general serological laboratory have been developed. It is recommended that a series of twofold dilutions of serum be used and that the dilution causing 50 per cent hemolysis be taken as the end point (either for estimating combining power of SLO or for determining ASLO titer). Experimental evidences are presented to support the recommendations.

Gratitude is due to Prof. John R. Paul for his guidance and counsel in this investigation. Thanks are also due to Mrs. Janet R. Copeland and Mrs. Helen S. Cadoret for their technical help.

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ULTRA-MICRO ROTATING STIRRER

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IN CARRYING out ultramicro titrations, the Linderstrom-Lang group in the Carlsberg Laboratory in Copenhagen have used an electromagnetic flea to mix the solution during titration.[1] A similar stirring arrangement was also later described by Claff.[2] Other investigators have accomplished mixing by bubbling air through the solution being titrated. In the apparatus herein described the microtest tube or beaker in which the titration is being carried out is rotated about the buret tip. This method has been found to provide adequate mixing. Its main advantage is that it eliminates the use of a bubbling tube or an electromagnetic bead, and thereby avoids the time and trouble of washing an additional piece of apparatus between titrations. The mixer herein described also provides a simple and versatile microtest tube and microbeaker holder, the height of which can be readily adjusted.

APPARATUS*

The test tube, or small beaker (see Fig. 1, *A*), is held in place in the holder, *B*. This holder is made up of two disks and two sets of springs. Each set of springs is made up of three individual springs arranged in the form of an equilateral triangle. One set of springs, *C*, is inserted into *D*, the top disk of the holder, and a similar set of springs, *E*, are inserted in the bottom disk, *F*. Disks *D* and *F* are in turn pressed into the metal tubes, *G* and *H*. The two sets of triangular springs accommodate tubes of various sizes and align them concentrically with the axis of rotation. The two metal tubes, *G* and *H*, slide up and down along the two vertical rods, *I* and *J*. These rods are attached to the rotating pulley (or disk), *K*. They are bent slightly from the vertical axis so that they are slightly divergent and thereby the rods, *I* and *J*, retain the holder *B* at any desired position. A concentric hole is drilled through the rotating disk, *K*, and this provides additional clearance for inserting microtubes (while the stirring apparatus is in position under the buret tip). The disk, *K* (see Fig. 2), rotates on a double ball bearing, *L* and *M*, and it is driven by the pulley, *N*, and the spring belt, *O*. The motor,† *P*, rotates at 1550 r.p.m. at full load and has a torque of 0.05 pounds per inch.

TABLE I

SELECTOR SWITCH POSITION	SERIES RESISTANCE (OHMS)	APPROXIMATE SPEED (R.P.M.)
0	∞	0
1	1300	150
2	1150	300
3	950	420
4	750	650

From the Department of Anatomy, Western Reserve University School of Medicine.
Aided by a grant from the National Foundation for Infantile Paralysis.
Received for publication, June 8, 1951.
*Available through the Micro-Metric Instrument Company, 7929 Kinsman Avenue, Cleveland 4, Ohio.
†Model YAA 711—2 Barber-Coleman Company, Rockford, Ill.

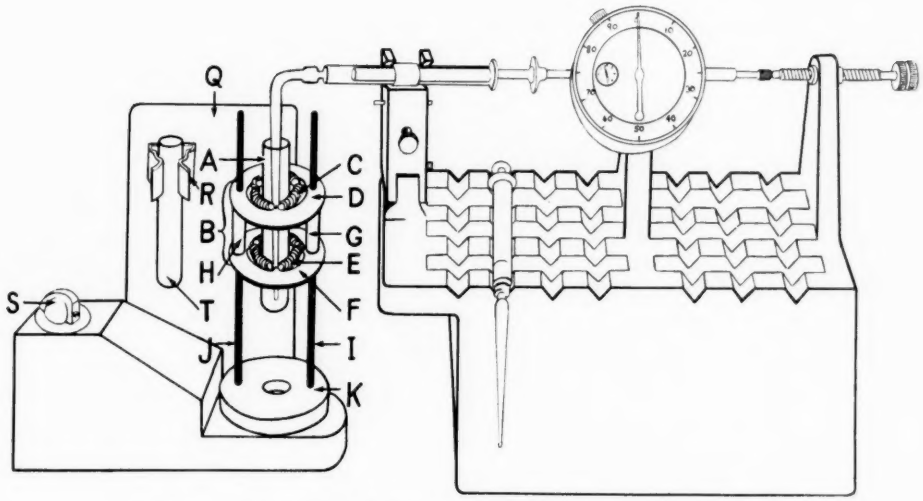

Fig. 1.—Micro rotating stirrer centered about syringe buret.

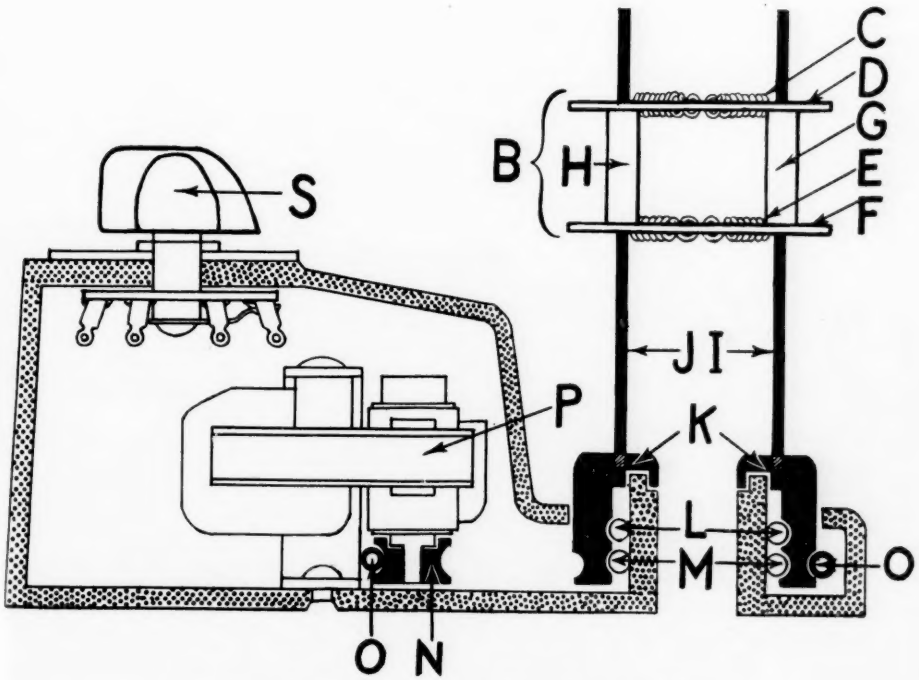

Fig. 2.—Details of construction of micro rotating stirrer.

The speed of rotation is geared down to one-third by the pulleys, *K* and *N*, and the speed of rotation is further slowed by using one of the series resistors shown in Table I. These are wired (not illustrated) to the selector switch, *S* (see Fig. 2).

The use of the microrotating stirrer with the syringe buret is shown in Fig. 1. The buret tip, which must have an exact 90 degree bend, is aligned in a vertical axis with respect to one of the vertical rods, *I*. The axis of rotation of the stirrer is adjusted concentrically with respect to the vertical axis of the buret tip by sliding the stirrer on the table. With the holder, *B*, in its lower position, the microtest tube (or beaker) is inserted through the two sets of "triangular" springs.* The holder *B* and contained tube is then raised about the buret tip until the latter is immersed into the liquid. If the diameter of the tube permits, it is preferable to center the vertical axis of the buret tip slightly eccentric to the axis of rotation. This accomplishes more effective stirring. The desired speed of rotation is obtained by inserting the appropriate resistor in series with the motor by means of the selector switch, *S*. Using a microtest tube (8 mm. I.D.) a rotation speed of about 400 r.p.m. insures adequate mixing.

Microtitrations are carried out in the usual manner. Excellent visibility of the end point is obtainable while the tube is rotating. A metal plate, *Q*, with a white background is attached to the back of the rotating stirrer to aid in the visualization. A clip, *R*, holds a comparator test tube, *T*, thereby permitting a precise determination of the end point. The mixer has been used for titrating gamma quantities of nitrogen with excellent results.

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Erratum

In the paper by George Gee Jackson, M.D., and Maxwell Finland, M.D. entitled "Turbidimetric Method for Determining Sensitivity of Bacteria to Antibiotics" (*J. Lab. & Clin. Med.* **38**: 289, 1951), on page 290, line 4, "490 λ" should read "490 mμ"; page 291, line 7, "490 λ" should read "490 mμ"; and page 293, legend to Fig. 2, "490 λ" should read "λ = 490 mμ."

*With long digestion tubes (50 mm.) it may be preferable to insert the tube in the holder B before centering the microstirrer about the buret tip.